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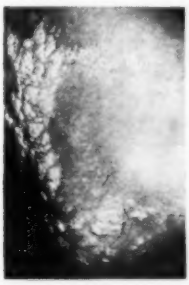

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Studies on Connective Tissue

III. Enzymatic Studies on the Formation and Nature of the Carbohydrate Intermediate of the Connective Tissue Polysaccharides in the Human Umbilical Cord

MELVIN D. SCHOENBERG, Ph.D., and RICHARD D. MOORE, M.D., Cleveland

Recently there has been increased emphasis on the biochemistry and physiology of the fibroblasts in normal and diseased tissues. The fibroblast is apparently a polyfunctional cell, since it is involved in the production of both the fibrillar elements and the matrix of connective tissue. Knowledge of the exact method of elaboration of the fibers and the ground substance and their relationship to each other and to the fibroblast is still incomplete. A possible insight into the mechanism of production of the polysaccharides of the ground substance is suggested from studies on the human umbilical cord.

We have reported the presence of a carbohydrate determined by histochemical methods, which may be an intermediate in the synthesis of hyaluronic acid and/or chondroitin sulfuric acid,[1,2] in the Wharton's jelly of the mature and the immature umbilical cord. The carbohydrate was abundant in the extracellular spaces of the most immature cords and gradually decreased in amount with increasing age of the cords, until only a small quantity could be demonstrated in term cords. The difference in concentration of this material and the differences in histochemical reactions of the mature and immature cords show that part of the synthesis of hyaluronic acid and/or chondroitin sulfuric acid is extracellular.

The carbohydrate intermediate is not a substrate for testicular hyaluronidase or α-amylase. It is insoluble in water and ethanol and is not removed by pyridine extraction. It is converted to a metachromatic substrate by esterification in the cold with sulfuric acid.[3] A material with similar characteristics has been described in the intervertebral disks of human embryos [4] and in some of the tissues and cells of the central nervous system.[5-8]

The present report is concerned with further enzymatic studies to determine the nature of the carbohydrate and its site of elaboration in the tissue.

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From the Institute of Pathology, Western Reserve University School of Medicine, Cleveland (Present address of both authors), and the Department of Pathology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

Methods

Human umbilical cords of various ages from six-week fetuses to term infants were used in this study. Cord age was determined from the size of the fetus. Frozen sections of 15μ were prepared from formalin-fixed tissue, except when lipid extractives were used in combination with Sudan black and Sudan IV, in which case Bouin's solution was employed.[9]

Metachromatic staining was done with aqueous solutions of twice-recrystalized toluidine blue (National Aniline Division, Allied Chemical Company, Certified Stain No. NU 12). Dye solutions, 1.5×10^{-4} M, were prepared in various buffers from pH 2.0 to 7.5 at intervals of 0.5 pH units and at ionic strengths of 0.0025, 0.01, and 0.1.[7,10] All sections were stained for 20 minutes. The above staining method was used after each of the following procedures:

1. An untreated control section
2. Hyaluronidase digestion
3. Sulfate esterification
4. Hyaluronidase followed by sulfation
5. Sulfation followed by hyaluronidase
6. α-Amylase digestion
7. α-Amylase followed by sulfation
8. β-Glucuronidase digestion
9. β-Glucuronidase followed by sulfation
10. Sulfate esterification followed by β-glucuronidase
11. β-Glucuronidase followed by hyaluronidase
12. β-Glucuronidase followed by hyaluronidase then sulfated
13. Hyaluronidase followed by β-glucuronidase
14. Hyaluronidase followed by β-glucuronidase then sulfated

Sulfate esterification was by the method previously reported.[8] Enzymatic digestion with β-glucuronidase (Nutritional Biochemicals Corporation) was according to the conditions presented by Fishman.[11,12] The enzyme solution was made in acetate buffer at pH 4.5, ionic strength 0.12, and contained 15,000 Fishman units per liter. The sections were incubated at 37 C for one hour and washed several times in 0.12 M NaCl and then in distilled water. Enzymolysis with testicular hyaluronidase (Nutritional Biochemicals Corporation) was done in 0.12 M NaCl at an average pH of 5.8 for 24 hours at 37 C. Alpha-amylase (Nutritional Biochemicals Corporation) digestion was according to the method described by Pearse.[9] In each of the solutions described above the sodium ion was the only metal ion employed.

If the sections were allowed to dry after the above procedures, they were hydrated through various dilutions of alcohol (ethanol) to water (95%, 80%, 70%, 50%, 25%, 5% ethanol to distilled water) and then dialyzed for two hours in the appropriate buffer.

In addition to these procedures the periodic acid-Schiff (PAS) method[13] was used before and after each of the following: amylase, hyaluronidase, and β-glucuronidase digestion and pyridine extraction of the tissue sections for 24 hours at 60 C. Sudan black and Sudan IV staining was also done before and after each of the above procedures.

Results

The metachromatic properties of untreated sections of all cords were identical to those already described.[1,2] Hyaluronidase digestion, sulfate esterification, hyaluronidase followed by sulfation, sulfation followed by hyaluronidase digestion, amylase, and amylase followed by sulfation gave the results previously reported.[1,2]

The Wharton's jelly of untreated sections, except those from the six- to seven-week group, stained metachromatically from pH 4.0 to 7.5. For a given pH metachromasy was more intense at the lower ionic strength, $\alpha/2=0.0025$. In the six- to seven-week cords no or only very slight metachromasy was observed. Below pH 4.0, the cords from six- to seven-week-old fetuses did not stain; however, older cords showed a faint basophilia of the interfibrillar areas in this pH range.

Sulfate esterification prior to enzymatic digestion produced a metachromatic material in Wharton's jelly at pH 2.0 to 4.0 and augmented metachromasy from pH 4.0 to 7.5 at all ionic strengths and in all cords. This response was greatest in the younger cords and diminished as the age of the cords increased.

Cords treated with testicular hyaluronidase did not give the metachromatic reaction at any pH or ionic strength. When hyaluronidase digestion was used before or after sulfate esterification, it had no effect on the metachromasy produced as a result of sulfation but still removed the original metachromatic material usually observed between pH 4.0 to 7.5.

Enzymolysis with β-glucuronidase gave different results. In the six- to seven-week group incubation with β-glucuronidase completely removed the material which could be made metachromatic by sulfation. This was in contrast to the results in this age group when hyaluronidase was used alone. In all instances sulfation prior to digestion with β-glucuronidase inhibited the enzymatic attack. The results were the same as obtained for sulfated non-enzyme-treated sections.

In the 8- to 17-week group treatment with β-glucuronidase did not effect the metachromatic properties of nontreated or hyaluronidase-treated sections. When β-glucuronidase was used before sulfation, the metachromasy usually produced from pH 2.0 to 4.0 was absent and the augmentation of metachromasy above pH 4.0 was not observed.

The series between 6 months and term closely paralleled the 8- to 17-week group. As with the latter, β-glucuronidase did not effect the usual metachromasy from pH 4.0 to 7.5 in Wharton's jelly. It differed from the preceding in that there was a slight residual metachromatic response from pH 2.0 to 4.0 after the β-glucuronidase and sulfate esterification procedure.

In order to determine whether the metachromatic reaction observed in the more mature cords at pH 2.0 to 4.0 resulted from the sulfation of hyaluronic acid and/or chondroitin sulfuric acid, dual enzymatic procedures were done. Hyaluronidase digestion, then β-glucuronidase digestion followed by sulfation, and the reverse, β-glucuronidase, then hyaluronidase digestion followed by sulfation, were carried out on sections from all cords. The dual treatment removed all the metachromatic material and potential metachromatic material which could be demonstrated by esterification under all staining conditions.

Although it has been shown that the sulfation procedure used here did not render glycogen metachromatic [3] sections were incubated with α-amylase prior to staining or sulfate esterification. Amylase and amylase digestion followed by sulfation did not effect the metachromatic response in the extracellular spaces.

Wharton's jelly was only faintly PAS-positive and was reversed with α-amylase digestion. Hyaluronidase and β-glucuronidase had no effect on the PAS reaction in the extracellular spaces. The fibers in this area were strongly PAS-positive and were not appreciably changed by digestion with α-amylase, testicular hyaluronidase, or β-glucuronidase.

There was no evidence of lipid in the extracellular spaces or in the fibers by the Sudan IV or Sudan black procedures.

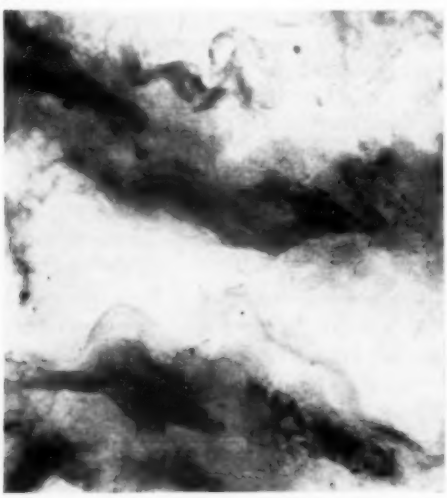
The metachromatic properties of the vessels in untreated, sulfated, and hyaluronidase-treated sections were as previously described.[1,2] The normal metachromasy throughout the vein wall was extended by sulfation to pH 2.0, as compared with pH 4.0 to 7.5 prior to sulfation. In the cords from five months to term the arteries were metachromatic in the outer one-half to two-thirds of the wall from pH 4.0 to 7.5 before sulfation. The inner one-third to one-half of the walls of the arteries in this age group were metachromatic from pH 2.0 to 3.0 to 7.5 in untreated sections. No enhancement of metachromasy was noted after sulfation in this latter area, but the outer portions of the walls showed an extension of the metachromatic reaction to pH 2.0. This was similar to the effect noted in Wharton's jelly and the vein wall. Here again, as with Wharton's jelly, the enhancing effects of esterification diminished with cord age, until it was slight or absent in term cords. In the cords below five months, where metachromasy was present throughout the arterial walls but confined to the pH range from 4.0 to 7.5, sulfation both augmented metachromasy and extended the pH range from 2.0 to 7.5. The arteries in the younger age group showed no concentration of metachromatic material in the subendothelial region either before or after sulfation. As was the case with Wharton's jelly, the metachromasy from pH 2.0 to 4.0 which resulted from sulfate esterification was uneffected by hyaluronidase. In term cords little or no metachromasy could be observed after the combination of hyaluronidase and sulfation. In the more immature cords there was a diminishing effect of hyaluronidase.

Beta-glucuronidase had no effect on the normal metachromasy of the vessels; however, the substrate in the younger cords which could be made metachromatic by sul-

fation was almost completely removed. In term cords β-glucuronidase in combination with sulfate esterification had very little if any effect on the metachromatic response of the vessel walls. From five months to term the effect diminished as the cord age increased. Below five months β-glucuronidase incubation prior to sulfation prevented metachromasy between pH 2.0 and 4.0. The metachromasy normally present was uneffected. In cords of the six- to seven-week group no metachromasy was noted in the vessel walls at any pH or ionic strength after digestion with β-glucuronidase and subsequent sulfate esterification. The dual treatments of hyaluronidase β-glucuronidase and the reverse negated the metachromasy of the vessels in all cords.

Amylase digestion with and without sulfation did not alter the metachromatic reaction in any area of the vessel walls. The PAS reaction was only faintly positive or negative in the regions corresponding to the location of the metachromatic material. Amylase digestion removed all the PAS-positive material in the metachromatic sites. Hyaluronidase and β-glucuronidase did not effect the PAS reaction in the vessel walls. The fibers were strongly PAS-positive and

Fig. 1.—Fibroblasts from a term umbilical cord with PAS-positive granules within the cytoplasm; × 1000.



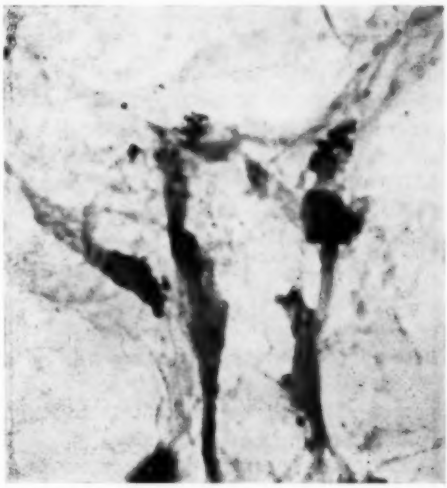


Fig. 2.—Fibroblasts from a term umbilical cord with Sudan black-positive granules within the cytoplasm; × 1000.

were not changed by amylase, hyaluronidase or β-glucuronidase. The positive material that was present in the muscle cells was removed by α-amylase.

The fibroblasts in all cords contained numerous, small, fairly uniform PAS-positive granules within their cytoplasm (Figs. 1 and 2). These granules varied in number within the cells of the same cord and from cord to cord, but in general more were present in the fibroblasts of the immature cords. In untreated sections the granules did not stain with toluidine blue at any pH or ionic strength. Following sulfation some of the granules became metachromatic, and the number of metachromatic granules increased with the age of the cord. Amylase and hyaluronidase digestion before and after sulfation had no effect on these granules. Beta-glucuronidase digestion removed most of them. As with the material in Wharton's jelly and in the vessel walls, sulfation prior to β-glucuronidase digestion prevented the action of the enzyme on the substrate. In these respects the two substrates appear identical.

Many of the granules within the cytoplasm of the fibroblasts were bright red by the PAS method (Fig. 1). In contrast to

the metachromatic granules in the mature cords, the PAS-positive granules were more numerous in the immature cords. They reached a peak at approximately four months and progressively diminished in number to term. Amylase digestion removed most of the PAS-positive material from the cytoplasm of the fibroblasts of the immature cords. With increasing age of the cord less material was removed by amylase digestion, so that at term most of the PAS-positive granules were still present. Hyaluronidase had no effect on the granules which remained after amylase digestion. Some of the granules were positive with Sudan black. Generally the number of granules which stained positive with Sudan black did not parallel those which were positive with the PAS reaction before amylase digestion. They tended to correspond to the distribution of granules which could be made metachromatic by sulfation or which were still PAS-positive after amylase digestion. The Sudan black-positive granules were not affected by amylase or hyaluronidase.

When the tissues were treated with β-glucuronidase after amylase digestion the PAS reaction was negative and the Sudan black staining was markedly diminished. Pyridine extraction of Bouin's fixed tissue had no effect on any of the staining methods which demonstrated the granular material.

Sudan IV-positive material was occasionally present in fibroblasts of the term cords. Extraction of the tissue with alcohol or pyridine for short times completely removed this material. No evidence of Sudan black or Sudan IV material was found outside of cells.

The mast cells in all instances were as previously described.[1,2] They were metachromatic over the entire pH ionic strength range in all cords above 13 weeks and were uneffected by the procedures used. In the youngest cords mast cells were rarely discernable in untreated sections. Sulfate esterification produced a number of metachromatic cells which cytologically corresponded to the mast cells of the more mature cords. Hyaluronidase, β-glucuronidase, amylase digestion, and pyridine extraction before and after sulfate esterification had no effect on these cells.

Comment

In several previous reports a carbohydrate intermediate of the connective tissue polysaccharides, hyaluronic acid, and/or chondroitin sulfuric acid has been suggested. It appears to be synthesized by the fibroblasts and elaborated into the extracellular spaces. Consequently, the final construction of hyaluronic acid and/or chondroitin sulfuric acid is probably extracellular; however, the metabolic mechanisms of this step are still unknown.

The concentration of precursor is high in Wharton's jelly and the vessel walls of the young cords and progressively diminishes as the age of the cords increase.

Only two types of cells can be identified in Wharton's jelly, fibroblasts and mast cells. It seems reasonable that one or both of these cells participates in the manufacture of hyaluronic acid. The following evidence excludes the mast cell. As a function of age, Wharton's jelly and the vessels are metachromatic before mast cells. From this it would seem unlikely that the mast cell completes a metachromatic polysaccharide before elaboration into the extracellular spaces. The potential metachromatic substrate of the immature mast cells which can be made metachromatic by sulfation would be the logical precursor elaborated into the extracellular spaces from these cells. However, the carbohydrate intermediate present in the extracellular spaces differs from the potential metachromatic substrate of immature mast cells in that it is sensitive to β-glucuronidase. Furthermore, it should be noted that mast cells in Wharton's jelly are largely confined to the immediate vicinity of the vessels.[14]

It seems more reasonable that the fibroblasts are the synthesizing cells of the connective tissue polysaccharides. Within the cytoplasm of the fibroblasts there are

numerous granules. These are similar to the granules reported by Gersh and Catchpole [15] in this location. From our results a large fraction of these granules contain glycogen. These are more numerous in immature cords and may indicate a more active metabolism of the cell during the period when cord growth is rapid. The decrease in granules containing glycogen in older cords suggests a less active metabolism of the connective tissue polysaccharides as tissue demands for growth diminish. In addition, some of the granules have many of the histochemical characteristics of the extracellular carbohydrate intermediate. They are metachromatic after sulfation and resistant to hyaluronidase and amylase digestion and are a substrate for β-glucuronidase. They differ by being positive with Sudan black. However, β-glucuronidase alters the granules sufficiently so that they do not take up or take up very little of the Sudan stain. It is unlikely that lipid is present. Prolonged extraction of Bouin's fixed tissue with pyridine at elevated temperatures has no effect.[9] The intracellular granules also differ from the extracellular material in terms of the PAS reaction. This test is negative or faintly positive in Wharton's jelly after treatment of the tissue with α-amylase, while some of the intracellular granules are still strongly positive. This could represent a concentration effect or a structurally different polysaccharide. After digestion with β-glucuronidase the amylase-resistant PAS-positive granules are no longer present, so that apparently the granules which can be made metachromatic by sulfation and which are Sudan black- and PAS-positive are similar.

The quantity of intracellular material which can be made metachromatic by esterification increases with the age of the cord and decreases in the extracellular spaces. This may indicate the metabolic state of the cell. The major role of the fibroblast appears to be synthesis, maintenance, and repair of the connective tissue components. When growth is rapid, the fibroblast furnishes a large amount of connective tissue polysaccaride precursor with very little accumulation in the cytoplasm. When growth slows down, it is presumed that the fibroblast is a reservoir of this material.

Other evidence has been reported in favor of the active part of the fibroblast in the synthesis of the connective tissue polysaccharides. Grossfeld, Meyer, and Godman [16] and, more recently, Castor [17] have found polysaccharides in tissue culture media of fibroblasts and synovial cells. The latter author has shown granular material within the cytoplasm of the cultured synovial cells, though he has not characterized these granules. Taylor and Saunders [18] have described a hyaluronidase-sensitive metachromatic material within the cytoplasm of the fibroblasts of granulation tissue. Wegelius and Asboe-Hanson [19] have described the uptake of metachromatic granules from disrupted mast cells by macrophages in early inflammatory processes. These observations of polysaccharides within cells of connective tissue are both in agreement with and different from our observations. The conditions under which metachromatic material has been identified in fibroblasts and related cells before release are different from ours. When injury is the stimulus for production of connective tissue polysaccharides, they could be completed in the cells before release, and therefore metachromatic material in the early repair stage may be recognized in these cells. The failure to recognize metachromatic material within these cells after granulation tissue has become well established would indicate a transitory capability of the cell to synthesize a more complete polysaccharide. On the other hand, as has been suggested by Wegelius and Asboe-Hansen,[19] this may be phagocytized high-molecular-weight polysaccharide from disrupted mast cells. There is no reason to limit phagocytosis to the granules of the mast cell. The fibroblast and/or macrophage could just as readily phagocytize hyaluronic acid and/or chondroitin sulfuric acid or other debris released as a result of the injury.

We have found carbohydrates within the cytoplasm of connective tissue cells of the umbilical cord; however, these carbohydrates were not metachromatic except in the mast cell. Developmentally, our results indicate that there is an elaboration of a much simpler nonmetachromatic polysaccharide into the ground substance and its completion extracellularly. The same pathway may also be followed in injury. The appearance of high-molecular-weight metachromatic polysaccharides in injury within the cytoplasm of cells may indicate a re-use of this material or a stimulus to cell reaction. Lattes, Martin, Meyer, and Regan [20] reported stimulation of the reparative process in cortisone-treated animals by the local injection of preparations high in connective tissue polysaccharides.

From distribution studies of the uptake of radioactive sulfur in soft tissues of developing animals and healing wounds of normal and scorbutic animals, the fibroblast uptake is far less than that of the extracellular material.[21] This would indicate that the sulfation is predominantly extracellular. Robertson and Hinds [22] reported concentrations of polysaccharide five times higher in wounds of scorbutic guinea pigs than in normal animals. Penney and Balfour [23] could not find metachromatic material in the healing wounds of scorbutic animals. After injection of ascorbic acid, metachromatic material was present in moderate amounts in the extracellular spaces at the end of 6 hours and in large quantity at the end of 12 hours. They, as well as Bunting and White,[24] described the presence of metachromatic material within the cytoplasm of the fibroblasts. This material was removed by ribonuclease, but not hyaluronidase. The extracellular material was sensitive to hyaluronidase, but not ribonuclease. They concluded that the intracellular metachromatic material is ribonucleic acid. In view of the work of these authors,[21-23] it would seem likely that the fibroblasts elaborate low-molecular-weight polysaccharide into the extracellular space. It is also suggested from the work of Robertson and Hinds [22] and Penney and Balfour [23] that the final synthesis of a high-molecular-weight polysaccharide is one of the basic defects in scorbutic animals. Once ascorbic acid (vitamin C) is replaced, total synthesis is accomplished rapidly in the extracellular location. It has also been suggested that ascorbic acid functions in the production of the fibrillar matrix.[25]

From our experiments we have concluded that the final construction of the high-molecular-weight polysaccharides, hyaluronic acid, and/or chondroitin sulfuric acid is probably extracellular in the human umbilical cord. The final elucidation of whether the fibroblast in all locations and situations is committed to only the elaboration of a polysaccharide precursor remains to be determined. At the present time this intermediate has not been isolated, but from the histochemical and enzyme studies it is possible to draw some tentative conclusions concerning the structure of this material.

The metachromatic behavior of the intermediate after sulfation clearly shows that it is not a protein, although it may be a part of a protein-polysaccharide complex. The results of esterification and β-glucuronidase sensitivity demonstrate the carbohydrate nature of this material. Since the substrate is attacked by β-glucuronidase, glucuronic acid is probably the nonreducing terminal sugar. Linkage to the following sugar would have to be in the β-configuration.

If the intermediate is a precursor of hyaluronic acid and/or chondroitin sulfuric acid, and since the nonreducing terminal sugar is glucuronic acid, further elaboration of the structure can be suggested from the work of Meyer and his group.[26-33] Beta-1,3-glucopyranosyl-2-desoxy-glucosamine is the repeating disaccharide unit of hyaluronic acid,[34-36] and β-1,3-glucopyranosyl-2-desoxy-galactosamine, the repeating disaccharide unit of chondroitin sulfuric acid. The original nitrogenous disaccharide and their N-acetyl derivatives are resistant to hydrolysis by β-glucuronidase.[26] The potential metachromatic substrate which is abundant

in the immature umbilical cords before the appearance of hyaluronic acid and chondroitin sulfate and which decreases as the larger compounds become more abundant may be comparable to the repeat disaccharide units obtained from the breakdown of the parent compounds by Meyer and his group.[26-30] In support of this is the sensitivity to β-glucuronidase of the material in the tissue. The fact that this material is sensitive to β-glucuronidase does not suggest a different fundamental sugar structure but would indicate that these sugars are not acetylated and suggests that acetylation occurs later in the total synthesis of the hyaluronic acid and chondroitin sulfuric acid.

Based on the metachromatic reaction after sulfation, the carbohydrate demonstrated by our methods must be larger than a disaccharide. The formation of a metachromatic complex depends upon available, regularly spaced, anionic groups, with at least four such groups in sequence.[37] Therefore, the minimum number of sulfate groups which we have introduced is four. The intermediate in tissue is at least a trisaccharide or tetrasaccharide; otherwise no metachromatic response could be produced. The selective attack of β-glucuronidase places glucuronic acid as the terminal nonreducing sugar.[26] Consequently, with only the end glucuronic acid affected, the rest of the chain would remain intact, and metachromasy would result with sulfate esterification if the original chain were greater than a tetrasaccharide. From the known repeat identity of the high polymeric polysaccharides, the tetrasaccharide would presumably be a dimer of the above disaccharide, with the hexose amidinic linkage in the β-1,4 configuration. Any distinction between the dimer of hyaluronic acid and chondroitin sulfuric acid would not be apparent with our methods, so that either one or both could be present. Since there is no basophilia of the interfibrillar areas below pH 4.0 in the younger cords, it is also unlikely that there is any sulfation of the carbohydrate prior to the experimental esterification. If such were present, even in the absence of regular spacing, one would expect binding of the dye by the anionic groups and consequently basophilia in these sites. Basophilia does appear in those areas which can be made metachromatic by sulfation at pH 4.0 prior to esterification. The appearance of basophilia at this pH and in this site would be consistent with the dissociation of the carboxyl groups, and their binding of the dye. This would suggest that the carboxyl groups of the uronic acid residue are not co-valently employed in some other linkage. The apparent availability of the carboxyl groups also lends support to the presence of a tetrasaccharide composed of the dimer of the disaccharides identified by Meyer and his group. Since only two such sites would be available in such a tetrasaccharide, basophilia would result, but metachromasy without sulfation would not occur because of the limited number of regularly spaced binding sites for the dye.

Summary

In the human umbilical cord there is a carbohydrate precursor of hyaluronic acid and/or chondroitin sulfuric acid. This precursor is a tri- or tetrasaccharide, and is present in the cytoplasm of the fibroblasts and in the extracellular spaces. Glucuronic acid is the nonreducing terminal sugar molecule of this carbohydrate intermediate, and it is nonacetylated. At this stage the carbohydrate is apparently carboxylated but not sulfated. The steps in the final construction of hyaluronic acid and/or chondroitin sulfuric acid from this intermediate, which is elaborated by the fibroblast, are not known.

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The Interrelationship Between Cortisone and Parathyroid Extract in Rats

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The individual effects of parathyroid hormone and cortisone have been studied extensively. Excess parathyroid hormone causes changes in teeth [1] and bones, induces hypercalcemia and nephrocalcinosis.[2,3] Cortisone causes changes in bones [4-6] which are different from those induced by parathyroid hormone. It interferes with calcification [7] and is reported to predispose to the formation of renal stones in man.[8,9] Cortisone induces hypercalciuria [10-12] without causing hypercalcemia. On the contrary it may reduce normal serum calcium concentration [13] and has been used to depress the hypercalcemia of vitamin D poisoning,[14,15] infantile hypercalcemia,[16-18] and sarcoidosis.[19-22] It has been claimed that cortisone does not affect the hypercalcemia of human hyperparathyroidism.[1,23]

The present investigation was designed to study the interaction of cortisone and parathyroid extract in the blood, bone, teeth, and kidneys of rats.

Materials and Methods

White male rats weighing 140 to 160 gm. were used. Two studies, one of three and the other of six days' duration, were performed. In both the animals were divided in the following groups: Group 1—controls. Group 2—parathyroid extract (Eli Lilly and Company, hereinafter called PTE), 200 units injected intraperitoneally daily. Group 3—cortisone acetate (Cortone Acetate, Sharpe & Dohme), 10 mg. injected intraperitoneally daily. Group 4—both drugs in the same dosage as in Groups 2 and 3, given intraperitoneally daily at the same time from separate syringes and at separate sites. The rats were kept in individual cages and fed a constant amount of ground and screened chow * that was suboptimal. Thus it was assured that all the rats ate all of the food offered them. Distilled water was given ad lib. In the rats studied for three days blood was drawn with use of anesthesia from the abdominal aorta for chemical analysis before killing. Calcium was determined with a Coleman flame-photometer [24] (this method gave results 10% to 15% higher than the ordinary titrimetric analysis [25]); phosphorus, by the method of Fiske and Subbarow.[26] Serum alkaline phosphatase was measured in Bodanski units.[27] Serum citric acid was estimated by the micromethod described by Natelson et al.[28] The jaws and teeth, tibia, and kidneys were fixed in neutral 10% formalin for 24 hours and washed in water. The bones and the teeth for the rats in the three-day study were decalcified in formic acid-sodium citrate.[29] One upper incisor was sectioned frontally at four levels in all the rats, and in the six-day groups the second upper incisor was sectioned longitudinally, as was one of the lower incisors. For the latter sections the teeth were partly ground before decalcification. All the histological preparations were stained with hematoxylin and eosin.

Results

Weight.—The control rats on the average maintained their initial body weight. The rats treated with PTE lost an average of 5%, and the groups treated with cortisone alone or in combination with PTE, about 9% of their body weight.

Serum Values.—The serum values of calcium, alkaline phosphatase, and citric acid for the groups treated for three days are shown in the Table.

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* Purina Laboratory Chow manufactured by The Ralston Purina Co., St. Louis.

The Serum Values of Calcium, Phosphorus, Alkaline Phosphatase, and Citric Acid for Each Group of Rats Studied *

		Ca, Mg./ 100 Ml.	PO_4, Mg./ 100 Ml.	Alk. Phosphatase, Bodansky Units	Citric Acid, Mg./ 100 Ml.
Controls	No. determinations	7	6	6	5
	Mean	12.03	8.5	30.3	2.6
	S. D.	±0.66	±0.67	±7.08	±0.17
Parathyroid extract	No. determinations	6	5	5	6
	Mean	13.73	7.32	29.1	2.08
	S. D.	±0.47	±1.09	±2.56	±0.44
Cortisone	No. determinations	5	6	4 2	3
	Mean	10.51	7.9	21 60.1	1.07
	S. D.	±0.4	±0.59		±0.46
Parathyroid extract and cortisone	No. determinations	6	5	4	4
	Mean	12.02	7.86	22.27	1.12
	S. D.	±1.1	±1.34	±6.62	±0.26

* The values are expressed as the mean for each group and the standard deviation. Because of a dual response of the alkaline phosphatase in the cortisone-treated animals, this group has been split into two.

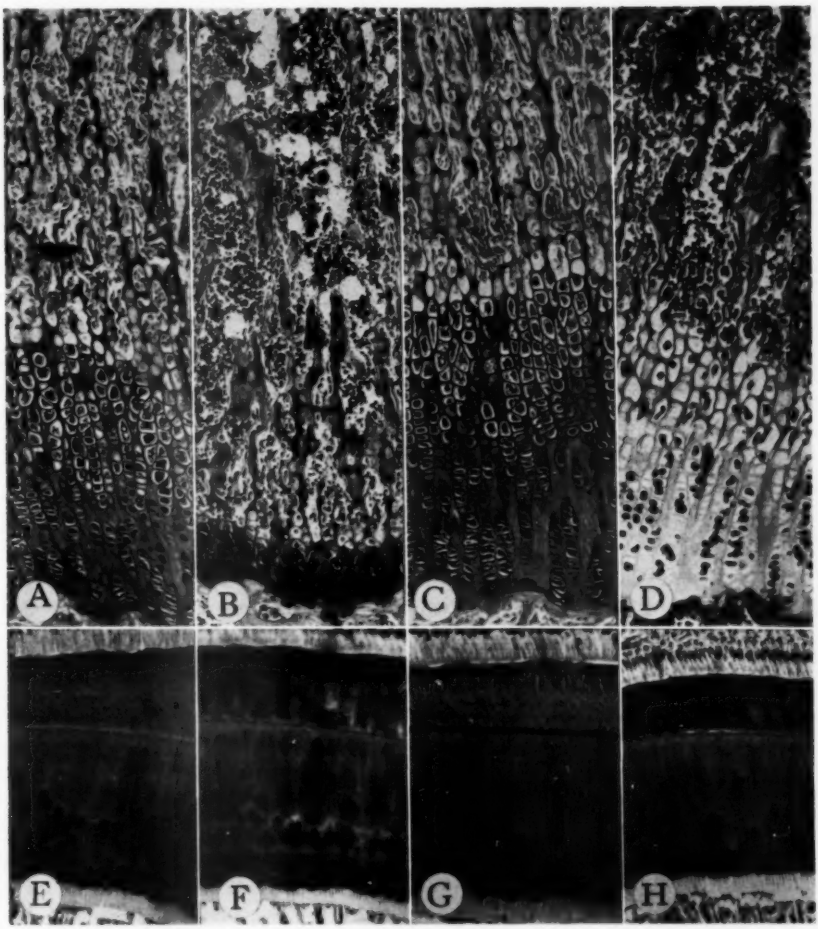
Calcium.—The mean serum calcium concentration of the PTE-treated group was significantly higher than that of the control group ($P<0.001$). Cortisone-treated animals had serum calcium levels significantly less than the control animals ($P<0.001$). When cortisone and PTE were given together the mean serum calcium approached the control values.

Phosphorus.—The mean serum phosphorus concentration of the control rats was 8.5 mg/100 ml. PTE, and cortisone had little influence on this value.

Alkaline Phosphatase.—The mean serum alkaline phosphatase in the group receiving PTE was not different from the value in the control group. Cortisone lowered the serum alkaline phosphatase concentration in four rats to 20.6, 21.1, 20.2, and 22.1 Bodansky units, respectively. In the remaining two rats the values were elevated to 61.2 and 59.0 Bodansky units. These ambivalent results were similar to the findings in larger groups of animals, which will be mentioned in the comment. The group which received both hormones had a lower mean serum alkaline phosphatase value than the control group, but this difference was not statistically significant.

Citric Acid.—Parathyroid extract did not affect the serum citric acid concentration. Cortisone alone or in combination with PTE lowered it significantly.

Histology.—Bones: The structure of the epiphyseal cartilage plate, the primary and secondary spongiosa in the tibia, and the membranous bones of the jaws were the object of attention in the bones. As the differences in the rats treated for three and six days are only degrees of intensity of effects, both groups will be described together. In the PTE treated animals (Figure, *B*) there was a widening of the epiphyseal cartilage with a thickening of the regularly arranged hypertrophic zone. The clear, vacuolated cells of this zone contrasted with the rather small and eosinophilic elements in this zone in the control animals (Figure, *A*). The epiphyseal-metaphyseal line was fairly regular. Resorption was poor, despite the presence of osteoclasts and the extensive proliferation of spindle-shaped cells replacing the usual rounded elements of the metaphyseal end. This accounted for the presence of broad cartilaginous cores and a thick dense primary spongiosa. The fibrous osteitis affecting the narrow malacic secondary spongiosa was reminiscent of the lesions observed in cases of hyperparathyroidism in man. Cortisone therapy (Figure, *C*) caused a marked thinning of the epiphyseal cartilage, affecting the proliferative as well as the hypertrophic zone. The epiphyseal-metaphyseal line was irregular. The broad dense primary spongiosa, with large cores of cartilage encased in the bony trabeculae, indicated decreased resorption. Osteogenesis was depressed, and the secondary spongiosa, poorly developed and markedly porotic. The osteoblasts were smaller and less numerous than in the controls. When the rats received both cortisone and PTE (Figure, *D*) the changes were as a whole similar to, but less pronounced than, those observed in the rats given PTE alone. In only one animal was the cortisone effect demonstrated by the narrowing of the epiphyseal cartilage and the irregularity of



Action of parathyroid extract and cortisone alone or in combination on the tibiae and teeth of rats after six-day treatment. *A*, longitudinal section through normal tibia. *B*, tibial section from PTE-treated animal. There is widening of the epiphyseal cartilage and a dense primary spongiosa. Spindle-shaped cells replaced the normal rounded elements between the trabeculae. *C*, tibia of cortisone-treated rat. Note marked thinning of the epiphyseal cartilage, encasement of cartilage in the trabeculae, and porosis of the spongiosa. *D*, tibial section from rat receiving both PTE and cortisone. The changes are similar but less pronounced than with PTE alone; × 105. *E*, frontal section of normal incisor. *F*, incisor after PTE administration. Layers of predentin alternate with layers of calcified dentin. *G*, incisor after cortisone administration. There is widening of the predentin. *H*, incisor from rat given injection of PTE, with cortisone administered simultaneously. Note rather normal appearance of the tooth; × 125.

the epiphyseal-metaphyseal line. Even in this animal the ratio of the proliferative to the hypertrophic zone of the cartilage and the fibrosis of the spongiosa were characteristic of hyperparathyroidism. The bones of the jaws reacted similarly to the metaphysis of the long bones and therefore will not be described separately.

Teeth: As a whole the teeth were less affected than the bones. The typical dental changes produced by one or both hormones are depicted in the lower part of the Figure. After PTE administration (Figure, *F*) there was a marked interference with the calcification of the dentin. Alternating layers of predentin and calcified dentin were

seen, the number of layers roughly corresponding to the number of days of treatment. Cortisone (Figure, *G*) caused a widening of the predentin but did not influence the uniform appearance of the dentin which was formed. The rats treated with cortisone and PTE (Figure, *H*) showed but little disturbance in tooth structure. Some teeth had a normal appearance; others had slight irregularities of the predentin-dentin junction. Occasionally an interrupted band of predentin was trapped in dentin. The enamel ameloblasts and odontoblasts were not distinctly affected by either treatment. Interestingly enough the changes described were more marked in the maxillary incisors than in the mandibular ones.

Kidneys: In the rats treated with PTE there was calcification of the kidneys which could be distinguished even macroscopically in the rats treated for six days. There was calcification with or without necrosis of the cells of the proximal tubules. Many calcified casts filled the distal tubular luminae. The kidneys of the groups treated with cortisone had a completely normal appearance with the methods used. This was also true when cortisone was given concomitantly with PTE, except in one animal, where two small intratubular calcified casts were seen.

Comment

The parathyroid extract used produced the changes expected from excess parathyroid hormone. There were typical bone changes, interference with normal calcifications of the dentin, hypercalcemia, and nephrocalcinosis.[1,2] This does not contradict the accepted belief that PTE has a direct action on bone calcium, causing it to shift into extracellular fluid.[3] The lack of significant PTE influence on serum phosphorus has been attributed in some reports to the present method of manufacture.[30-32]

Cortisone induced marked metabolic changes. It reduced the normal serum calcium level. This has been observed before in rabbits by Pincus et al.[13] One possible explanation of this is the increased urinary and fecal excretion of calcium following cortisone administration.[10,11,22] It is worth mentioning that increased concentrations of serum calcium were found after adrenalectomy in rabbits,[33] rats,[34] and dogs.[35] Taylor and Caven[36] found this to be true also in parathyroidectomized animals. Guleke[37] reported that adrenalectomy relieved parathyroprival tetany in animals. This has been reported in a number of human cases of hypoparathyroidism who developed adrenal insufficiency.[38-43]

Cortisone had a profound influence on the cartilaginous and membranous bones and teeth. The changes in the bones generally resembled those described by Follis.[4] There was a diminished activity of all the elements of the bone. The proliferation and resorption of the cartilage cells was decreased. The lessened bone formation explains the picture of osteoporosis. There was widening of the predentin, demonstrating a disturbance in the calcium deposition in the teeth. This has been previously described in treated rachitic rats.[7] The exact mode of action of cortisone upon calcified and calcifiable substance is still uncertain. The increased excretion of calcium and resulting hypocalcemia are not the only factors. A direct action of cortisone on the bones and teeth must be considered because of the variety of changes induced in the elements of these tissues. This belief is supported by the finding of hypercalcemia in cortisone-treated nephrectomized dogs.[44]

If both hormones were given simultaneously the results obtained demonstrated an antagonistic action between cortisone and parathyroid hormone. The mean serum calcium concentration was maintained at normal levels. The nephrocalcinosis was prevented, and the bone sections showed only minimal effects of hyperparathyroidism. This was true also for the teeth. In reported cases of human hyperparathyroidism and established hypercalcemia, cortisone has failed to reduce serum calcium levels.[14,23] An interesting phenomenon was the prevention of nephrocalcinosis in spite of an in-

creased urinary calcium excretion under the given conditions.[45] Bacon et al.,[46] in a recent study with radioactive calcium, also found that cortisone diminished the deposition of Ca^{45} in the kidneys of animals concomitantly treated with PTE. Engel[47] showed that PTE led to depolymerization of the ground substance of connective tissue. Baker et al.[48] described this change on ground substance as a prerequisite for renal calcification. On the other hand, cortisone is reported to increase the polymerization of mucopolysaccharides[49] and to decrease their production and serum levels.[50,51] Whether these findings are in favor of an antagonism based on opposing actions on a common substrate rather than an intermolecular ablation is not clarified by the present study.

The dual potential of cortisone in inhibiting or increasing serum alkaline phosphatase has been clearly demonstrated in a separate study.[52] The hypocalcemia induced by cortisone is known to be accompanied by a drop in the serum citric acid,[13] as also is the hypocalcemia caused by hypoparathyroidism.[53] Parathyroid extract given in high doses or over prolonged periods of time increases serum citric acid.[54,55] The dose and time factor in our experiment was apparently not sufficient to do this. When both hormones were administered together the parathyroid extract did not prevent the cortisone from lowering the serum citrate. Thus cortisone lowered the serum citric acid level even in the presence of normal serum calcium values.

Summary

Normal rats were given injections of parathyroid extract, cortisone, and both hormones together. The action of these hormones has been studied by chemical and histologic methods.

Cortisone has been found to antagonize some of the manifestations of parathyroid extract in the blood, teeth, bones, and kidneys.

The mode of action of cortisone on calcium metabolism and its interference with the parathyroid action are discussed.

Dr. Reidar F. Sognnaes, of the Harvard School of Dental Medicine, helped in this study. Mr. George Pettengil, from the same institution, prepared the sections of the teeth in the six-day study. Dr. Thomas Egan performed the calcium determinations, and Dr. Glen Irwin, Lilly Research Laboratories, Indianapolis, supplied the parathyroid extract.

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Hyaline-Membrane Disease in the Newborn Associated with a Lower Accessory Lung

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A case of hyaline-membrane disease in newborn infant associated with a lower accessory lung, or *Nebenlunge*, is presented with the belief that it is the first reported case of the coexistance of these entities. A brief review of the literature is presented, with special emphasis on the etiology of hyaline-membrane disease with the possibilities of its pathogenesis as related to the *Nebenlunge*.

Report of a Case

The mother was a 30-year-old Caucasian woman, tertigravida and secundipara, who had had two previous cesarean sections because of an outlet contraction. The first two siblings weighed 3700 gm. and 3800 gm., respectively, breathed spontaneously, and have developed normally since birth. From the beginning of the second trimester of this gestation there was minimal edema of the ankles, hands, and face, with occasional traces of albumin in the urine. There was no associated elevation of the blood pressure. The patient was treated with a low-salt diet and diuretics, and her total weight gain at term was 40 lb. In the 39th week a cesarean section was performed, and a 3150 gm. boy was delivered, whose condition was described as good. Thirty minutes later he was found to have a dusky pale color and labored respirations. Therapy consisted of an environment of high humidity and intranasal oxygen and intramuscular injection of 200,000 units of penicillin and 250 mg. of streptomycin. His dusky color and labored respirations persisted, and he developed sternal retraction. Transient symptomatic improvement appeared with changes in the oxygen concentration. After a progressively downhill course the patient died 15 hours 10 minutes after birth.

Pathological Findings

The autopsy was completed approximately 11 hours after death, and significant findings were confined to the thorax and abdomen. Grossly the lungs were the size of well-expanded infant lungs, but they were dark reddish-purple and had a liver-like consistency. The tracheobronchial tree and pulmonary vasculature were unremarkable. The heart and great vessels were within normal limits for this age group. An anomalous mass of tissue was found in the left thoracic cavity, resting on the superior surface of the diaphragm (Fig. 1). This accessory organ was freely movable and attached to the diaphragm by a hilum containing three vessels, two arteries and a vein. There was a 1.5 cm. defect in the diaphragm just lateral to the esophageal hiatus which allowed passage of these vessels into the abdomen. The entire organ was enveloped in a mesothelial tissue which was continuous with the parietal pleura. The arterial

Fig. 1.—The *Nebenlunge*, exposed in the otherwise eviscerated thoracic cavity. It is held forward by the forceps, and a probe is inserted into the esophagus.

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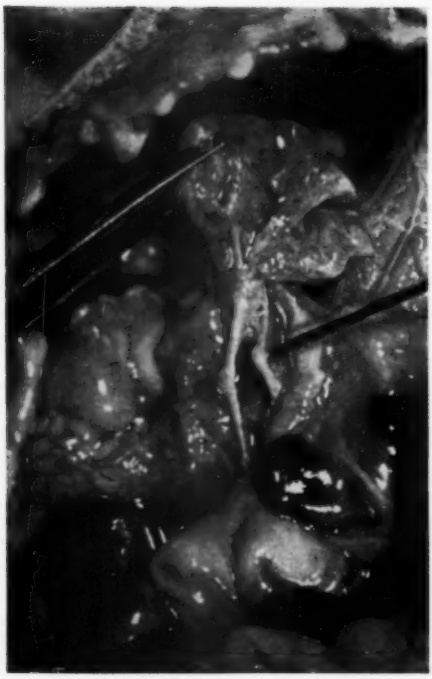

Fig. 2.—The *Nebenlunge* has been sectioned and is held by the forceps. The probe lies beneath the arterial supply. The vein cannot be visualized.

supply was twofold, one vessel arising from the base of the celiac axis and the other from the left gastric artery 1.5 cm. from its origin (Fig. 2). The venous drainage consisted of one small vein which emptied into the inferior vena cava just below the diaphragm. On section this tan organ had the consistency of atelectatic lung. No bronchial elements could be identified, and there was no connection to the upper respiratory tree.

Microscopic sections of the right and left lungs revealed marked vascular engorgement and a resorption atelectasis. The alveolar ducts and alveoli which remained open were lined by a homogenous eosinophilic material forming an adhesive membrane (Fig. 3).

Microscopically the accessory intrathoracic organ was composed of tissue of a pulmonary type, with bronchi and alveolar anlage in varying stages of development. The bronchiolar elements were tubular, with frequent invaginations of the wall, and were lined by a ciliated columnar epithelium. The organ was serially sectioned, and no cartilaginous element was identified. The alveolar spaces were lined by a low cuboidal epithelium. In a few areas the tissue was quite hypoplastic, consisting only of large numbers of bronchial elements and mesenchymal tissue containing sparse numbers of alveolar spaces. The remainder contained moderate numbers of bronchial elements and fairly well-developed cuboidal epithelial-lined alveolar spaces, the walls of which contained alveolar capillaries in varying stages of development, the mesenchymal tissue never being prominent. Found loose in the alveolar spaces were varying amounts of a stringy eosinophilic material (Fig. 4). In rare instances it was apparently adherent to the alveolar epithelium, but in no instance was there a complete membrane formation.

Histochemically the eosinophilic material of the right and left lung and the accessory lung were identical. Both materials were positive with the periodic acid-Schiff technique, yellowish-red with Mallory's phosphotungistic acid-hematoxylin, purple with methylrosaniline chloride (crystal violet), and metachromatic with toluidine blue.

Comment

Lower accessory lungs on the left and rarely on the right without connection to the upper bronchial tree have been described by anatomists for many years. Only recently, because of surgical implications, this rare developmental anomaly has become of clinical importance. Extensive bibliographies for most of the reported cases, now around 50, are available in the literature.[1-3] Included in the above cases are several in which the accessory lung was found in an intra-abdominal position. Most authors feel that the derivation of these intra-abdominal lungs is similar to that of the left lower accessory lung. The problem of derivation has given rise to three theories, the first and most commonly accepted being that the accessory pulmonary tissue is the result of sequestration of developing pulmonary tissue early in the course of the embryological development. Hammer [4] discovered in serial sections of a

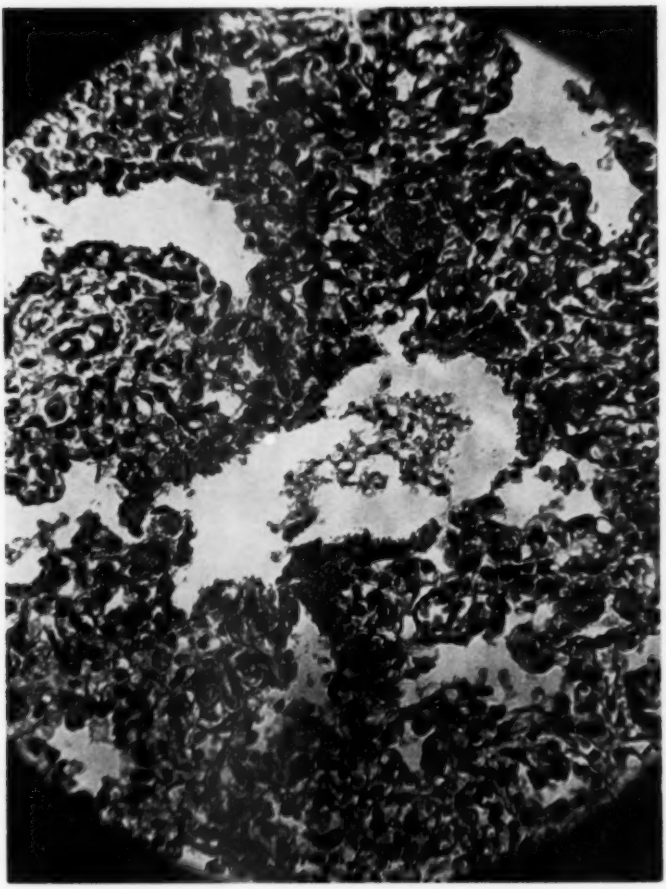

Fig. 3.—Lung parenchyma, demonstrating a central alveolar duct lined by eosinophilic membrane and surrounded by engorged vessels and areas of resorption atelectasis; reduced 8% from mag. × 200.

11.7 mm. embryo sequestration of pulmonary tissue. Wax-plate reconstruction revealed the underdevelopment of one of the bronchi which would have ordinarily supplied the involved area. Unfortunately, his findings were in an upper lobe rather than a lower lobe. In the second theory sequestration is also recognized as a factor but it is believed to be the result of adhesions between the lung blastema and the celomic mesothelium. The third theory involves the possibility of origin from the foregut due to a downward displacement of the cells from the tracheoesophageal ridge.[2]

The clinical findings in cases of left lower accessory lobe are minimal and in no way pathognomonic.[1,5] The commonest findings are those of bronchial compression by a tumor mass in the thoracic cavity. The literature describes no case in which the diagnosis was made preoperatively. Wachs[1] feels that malignant degeneration occurs more frequently in these *Nebenlunge* than in normal pulmonary tissue. Three cases of tuberculosis occurring in the accessory lung have been described.[6-8] In one of these cases there was a massive fatal intrathoracic hemorrhage secondary to tuberculous cavitation in the accessory lung found at postmortem examination. Accessory lungs lying in the pericardial cavity have been described and have resulted in fatal congestive failure.[1]

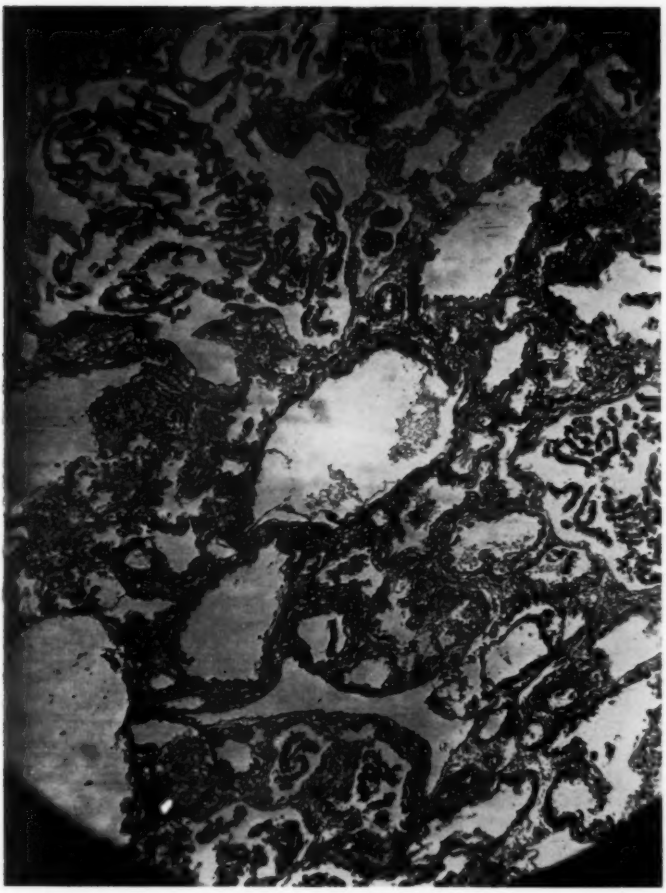

Fig. 4.—Microscopic section of the *Nebenlunge*, revealing in a central alveolar anlage precipitated eosinophilic material with similar histochemical properties to eosinophilic membrane in Figure 3.

Since Hoccheim[9] first described a peculiar membrane formation in the lungs of two newborn infants, a voluminous amount of literature has appeared on the subject now known as hyaline-membrane disease. Johnson and Meyer,[10] during the course of investigation of 500 autopsies on stillborn and newborn infants, were the first to actually describe the hyaline-membrane syndrome. They concluded that the hyaline membrane was derived from the aspiration of epidermal cells and the fat of the vernix caseosa. Farber and Wilson,[11] in 1932, were able to produce a membrane lining the alveolar ducts, atria, and alveoli by immersing rabbits in an India-ink solution for 60 to 90 seconds followed by a short period of dyspnea. Although complete and characteristic membranes were formed from the India ink, there was no associated vascular engorgement or atelectasis. These same authors succeeded in producing a membrane with the same histological and histochemical characteristics as that observed in newborn infants by injecting a fibropurulent exudate from a case of empyema into the trachea of rabbits and producing hyperventilation with a Drinker respirator. Again, although typical eosinophilic membranes were formed, no resorption atelectasis or vascular engorgement was present. They found they could reproduce the same findings in dead animals. Many investigations since that time have agreed that the essential pathogenesis is

primarily aspiration of the amnionic fluid content.[12-15] Tran-Dinh-De and Anderson,[16] as well as Farber and Wilson[11] in their earlier studies, emphasized the fact that similar membranes occur in many infectious diseases and intoxications. In 1949, Miller and Hamilton[17] were the first to dispute the etiological significance of aspirated anmiotic fluid content. They described epithelial injury in the alveoli and felt that the proteinaceous eosinophilic material was derived from the blood rather than aspirated amnionic fluid content. They also described the presence of an eosinophilic granular material in the alveolar spaces without membrane formation in the lungs of 4 of 32 stillborn infants. In a later study production of eosinophilic membranes was accomplished in rabbits by bilateral vagotomy.[18] Production experimentally of the eosinophilic membrane with associated vascular engorgement and resorption atelectasis was reported by Laufe and Stevenson.[19] This was accomplished in guinea pigs by the intratracheal installation of equal parts of amnionic fluid and plasma. They could not reproduce the syndrome by installation of equal parts of 5% thromboplastin and plasma and suggested the presence of some antihemorrhagic factor in the amnionic fluid not yet described.

The exact histochemical structure of the eosinophilic membrane in this syndrome has been the subject of much controversy. The usual histochemical techniques for demonstration of fibrin have always resulted in negative findings. Gitlin and Craig,[20] in their classical work with fluorescent-labeled antihuman fibrin antibodies, have demonstrated that the eosinophilic membrane is composed primarily of fibrin. Further studies revealed the explanation for the negative fibrin histochemical studies.[21] These investigators found that in the absence of an albumin concentration of greater than 1 gm/100 cc. and/or a glutathione concentration of greater than 0.05 gm/100 cc. in vitro fibrin prepared by the mixture of purified thrombin and fibrinogen resulted in negative histochemical studies for fibrin. Their fluorescent-labeled-antibody studies were positive, and it was concluded that the eosinophilic membrane was derived from blood proteins, as previously suggested by Miller et al.[17] In three cases well-defined eosinophilic membranes resulted in a positive fibrin stain. In the other two premature infants some fibrin-positive membranes were demonstrable, and in many unexpanded atria and alveolar ducts fibrin networks were found which, although positive for fibrin, did not form a membrane. These observations recall those of Miller and Hamilton,[17] in which they found a similar eosinophilic network on hematoxylin and eosin sections in stillborn infants. Studies of the lungs of human newborns who did not succumb to hyaline-membrane disease did not contain any fibrin in the alveoli or alveolar ducts.[22]

In the case reported herein, the staining characteristics are compatible with those described by most authors. The eosinophilic material found in the alveolar ducts and spaces of the accessory lung which had no connection to functioning respiratory tree gave similar staining reactions. We feel that, as in the cases of Miller and Hamilton[17] and Gitlin and Craig,[20] this material is a fibrin in the alveolar spaces which has not formed a membrane against the walls because of lack of a plastering action by the inspired air during respiratory activity. This is in accord with the India-ink studies done by Farber and Wilson.[11]

According to the work of O'Neal et al.[23] regarding the morphologic development of the pulmonary arterioles, it would appear that the media muscle mass theorized to be an important factor in the control of total pulmonary blood flow and pressure in intrauterine life could be responsible for the deposition of the fibrin precursor by enabling the easier transfer of blood proteins from these vessels into the alveolar spaces as a result of the "paucity of arteriolar media." This could in part, if true, explain the presence of the material loose within the alveolar spaces of our accessory lung.

Summary

A case of hyaline-membrane disease of the newborn associated with an accessory left lower lung without connection to the upper respiratory tree is presented. The accessory lung contained masses of eosinophilic material with a histochemical structure similar to that of the hyaline membrane found in the right and left lungs. It is felt that these findings could support the hypothesis that hyaline-membrane disease is the result of an exudation of a plasma fibrin precursor by the fetus.

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Septic Retinitis Due to Candida Albicans

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Introduction

Although mycotic infection of the eye by any route is rather infrequent, instances due to an endogenous metatastic spread appear exceedingly rare (Duke-Elder[1]). Of the agents responsible for the ocular mycoses, Candida albicans seems one of the less usual invaders. Early instances of conjunctival infection with C. albicans have been reported by Norton,[2] 1927, and Castellani,[3,4] 1928. Apparently the only previous instance of intraocular moniliasis was reported by Miale,[5] 1943. In this instance, panophthalmitis followed iritis and uveitis, and there is suggestion, at least, that the eye may have been infected by an exogenous route rather than by blood-borne infection.

The present case was considered of interest because in a patient suffering from a blood stream infection with C. albicans a small retinal lesion resembling a Roth spot appeared which at postmortem examination was found to contain the hyphal elements of the yeast. The increase in recent years of the recorded instances of systemic moniliasis suggests that further examples will come to light.

Nature, Variations, and Pathogenicity of C. Albicans

Although the nomenclature is still not completely uniform, it is now generally agreed that the yeast-like organism most usually associated with clinical thrush infection may properly be called C. albicans. Conant,[6] 1952, defines this as a yeast-like fungus of oval budding form, producing both pseudomycelia and blastospores in exudates and tissue, as well as in culture both at 37 C and at room temperature. The older terms, Oidium albicans (Rubin,[7] 1853), Monilia albicans (Zopf,[8] 1890) are considered as synonyms by Conant,[6] but the genus name Candida (Berkhout,[9] 1923) is preferred.

The ubiquitous distribution of C. albicans is generally well known. Lewis,[10] 1939, using an intradermal skin test with a commercial extract of C. albicans, found that 46% to 50% of normal persons gave a positive response. Direct culture has also resulted in the isolation of the organism with consistent frequency. In 100 normal children, McGovern et al.,[11] 1953, cultured C. albicans from the mouth in 14% and from the rectum in 6%. Including 100 persons in each series, Robinson,[12] 1954, obtained positive culture of this yeast from the stool, vagina, and oropharynx in from 14% to 16%. Similarly, Wegmann,[13], 1953, found C. albicans in about 10% of material from pharynx, colon, and vagina in normal persons.

In view of the widespread nature of subclinical infection with C. albicans, a question of great interest arises as to how the organism may change its mode of growth to become not only a surface but a systemic pathogen in man.

Since the morphological variations of C. albicans are a striking feature, the cause and significance of these changes may be considered. In vitro studies have shown that the morphology of the yeast is dependent, to some extent at least, upon its growth environment (Scherr and Weaver,[14] 1953). In the study limited to C. albicans, Wickerham and Rettger,[15] 1939, noted an apparent hereditary tendency for a predominance of

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hyphal or yeast-like forms in particular strains. However, in general, with highly nutritious media and sufficient oxygen, most strains grew almost entirely in the form of yeast-like cells. Oxygen deprivation enhanced hyphal and chlamydospore formation. Other factors favoring the hyphal (mycelial) phase include starvation media, liquid media in general, high pH, and high temperature (37 C) (Skinner,[16] 1947). More detailed studies of Nickerson,[17] 1950, on artificial media, indicated that the maintenance of the yeast phase was to a large extent dependent upon the presence of a sulfhydryl group.

It is possible that the precise factors responsible for variations in growth characteristics may be confused by strain differences within the species C. albicans. For example, Lewis,[10] 1939, reported marked inhibition of the growth of other micro-organisms by C. albicans, while Robinson,[12] 1954, found that simultaneous inoculation of the yeast and common flora (Escherichia coli, Aerobacter aerogenes, Staphylococcus albus, S. aureus) would eventually lead to overgrowth of C. albicans by the bacteria.

Dimorphism is apparently a constant feature of clinical infection. In sputum, either the yeast or mycelial form may predominate, or there may be a mixture of the two (Conant,[6] 1952; Moss and McQuown,[18,19] 1953). In tissue one gains the impression that the growth tends to be in mycelial form. The problem of identification here may be somewhat more complicated, since hyphal elements may be cut transversely and one may hesitate before identifying a rounded yeast-like element as being truly the yeast form of growth (Zimmerman,[20] 1950, and other references listed under the tissue reaction to C. albicans).

Much controversy has attended the discussion of the effect of antibiotics upon the growth of C. albicans.

Attention may first be turned to the study of the effects of antibiotics in vitro. Under these circumstances Woods et al.,[21] 1951, found that in Sabouraud's broth no apparent quantitative increase in the growth of C. albicans was apparent by the addition of chlortetracycline (Aureomycin), up to 100μg. per cubic centimeter; penicillin, up to 5 units per cubic centimeter, or chloramphenicol, up to 1μg. per cubic centimeter. Robinson,[12] 1954, also failed to find a quantitative increase in growth on Sabouraud's medium after the addition of penicillin, oxytetracycline or chlortetracycline, chloramphenicol, or erythromycin. On the other hand, Johnson,[22] 1954, reported that chlortetracycline supported the growth of C. albicans at an optimum pH of 5.1-6.4. Previously Pappenfort and Schnall,[23] 1951, had found that chlortetracycline for oral administration gave increased growth of C. albicans with use of diffusion-plate technique. They were inclined to believe that the growth-promoting factor was a substance in this drug separate from its antibiotic portion, since it was not inactivated by boiling for 10 minutes (which destroyed the antibiotic principle) and seemed absent in the chlortetracycline prepared for parenteral use. They were unable to find any increase in the growth of C. albicans by addition of bacitracin, chloramphenicol, penicillin G, streptomycin, oxytetracycline (Terramycin), and various vitamins to the media.

Experimental work concerning the effects of antibiotics upon C. albicans in vivo is apparently not extensive. The experience of Foley and Winter,[24] 1949, indicated that penicillin potentiated the pathogenicity of C. albicans in chick embryos, and they reported that two rabbits infected with yeast and treated with penicillin died before the controls. Confirmation of these findings would seem desirable. Using chlortetracycline, Seligmann,[25] 1952, noted that it increased the mortality in mice resulting from measured doses of C. albicans. As opposed to Pappenfort and Schnall,[23] 1951, he, however, noted the potentiating factor to be heat labile and inactivated by standing at room temperature in a fashion quite similar to the antibiotic properties of the drug. Since there appeared no modification of the patho-

genicity of C. albicans after passage through an animal treated with chlortetracycline, he concluded that the potentiating effect in vivo was probably due to lowering of host resistance by chlortetracycline.

The possibility that antibiotics themselves do not result in an increased incidence of moniliasis in adult human subjects suffering from short illnesses seems supported by Robinson's,[12] 1954, study in which after two to four weeks of antibiotic therapy of various types (including chlortetracycline) stool and vaginal and oral pharyngeal cultures showed no greater incidence of C. albicans than did the control series. Again, however, the evidence is conflicting, as in small children (with perhaps a somewhat different enteric flora) McGovern et al.,[11] 1953, found a definite increase in the incidence of C. albicans isolation from the bowel after chlortetracycline and chloramphenicol administration. It is of importance that 21 of the 45 children studied were considered well at the time the antibiotic was given. Despite the rather controversial nature of the effects of antibiotics upon the growth of C. albicans, there is considerable clinical opinion to support the thesis that prolonged antibiotic therapy is often attended by clinical infection with this yeast (Zimmerman,[20] 1950; Woods et al.,[21] 1951; Livingood and Mullins,[26] 1952; Brown et al.,[27] 1953). Chlortetracycline in particular has been pointed out as an agent prone to be associated with moniliasis (Harris,[28] 1950; Pappenfort and Schnall,[23] 1951).

If the growth-potentiating feature of antibiotics is set aside for the moment as a suggestive but not soundly proven feature, one's attention may be turned to a consideration of possible causes for the depression of the host defenses. In reviewing the case reports it is apparent that those afflicted with severe moniliasis, particularly the systemic form, tend to be those suffering from chronic debilitating illnesses. This suggests that whereas in the preantibiotic era these patients would have succumbed to a terminal bacterial infection they now, thanks to antibiotics, are preserved from such a death only to fall prey to the first organism capable of passing the body's routed line of defense. Kligman,[29] 1952, has aptly phrased this as the "opportunistic pathogenicity" of C. albicans. Zimmerman,[20] 1950, was impressed by the frequency with which systemic moniliasis accompanied the use of anti-folic-acid drugs. The frequent association of leukemia and moniliasis (Farber,[30] 1950; Zimmerman,[20] 1950) has been noted and may indicate the lack of some bone marrow factor. Zimmerman,[20] 1950, also pointed out the possibility that interference with normal intestinal flora by the administration of antibiotics may enhance the growth of C. albicans either by the lack of a specific inhibitory factor or by the lack of proper vitamin synthesis by the normal enteric bacteria. This tendency to sterilization of the bowel by oral antibiotics was confirmed in the work of McGovern et al.,[11] 1953, among others.

From a pathological viewpoint, the variation in tissue reaction to the presence of C. albicans is a striking feature of the reported cases. Brown et al.,[27] 1953, illustrate abscess formation in the brain, myocardium, and kidney, with a predominantly mononuclear tissue response surrounding the yeast. Moss and McQuown,[18,19] 1953, noted that the response may be in the form of mild to severe chronic inflammation. In the lungs the mycotic lesions may take a form resembling miliary tuberculosis. The nodules are small and yellow and may fuse together and break down to form cavities. The nodules are frequently necrotic at their center, where the hyphal threads may be seen surrounded by epithelioid cells, giant cells, and lymphocytes (Boyd,[31] 1950). On the other hand, the tissue response may be nearly lacking, as illustrated by Schaberg's,[32] 1955, Figure 2, showing the hyphae of C. albicans in a renal glomerulus with no apparent cellular reaction.

In the meninges, Halpert and Wilkins,[33] 1946, found hyphae of C. albicans both lying free in the subarachanoid space and surrounded by polymorphonuclear leukocytes.

Other hyphae had been engulfed by huge giant cells. This giant-cell response to the hyphae was also noted in the cases of Candida ependymitis reported by Miale,[5] 1943, and Morris et al., [34] 1945. The variation in inflammatory tissue response may be mirrored in the wide differences in the cell counts of cerebrospinal fluid of reported cases of monialiasis of the central nervous system (Fine et al.,[35] 1955).

Schattenburg and Flinn,[36] 1939, administered varying doses of C. albicans intravenously to rabbits. The cellular response was found to vary with the magnitude of the infection. Animals dying in 24 to 48 hours after the intravenous infection showed pulmonary lesions with central necrosis and surrounding polymorphonuclear leukocytes and only slight lymphocytic response. In instances in which death occurred from 10 to 21 days after infection the lesions in the lungs resembled pulmonary tubercles and showed central giant-cell and epithelioid-cell accumulations, with a peripheral zone of lymphocytes. Smith and Sano,[37] 1933, found that the pathogenicity in rabbits tended to vary apparently with equivalent doses in four different strains of C. albicans. This variation in the response to different strains may be a further reason for differences in the tissue response to infection.

Report of Case

Summary.—A 53-year-old housewife was admitted with multiple myeloma. Her course was complicated by anemia, enteritis, pneumonitis, hepatic insufficiency, and intolerance to prednisone therapy. Large doses of antibiotics were used. C. albicans was cultured early from the vagina. One month before death, it was obtained from urine, blood, pharynx, and bone marrow. At the time of systemic moniliasis a hemorrhagic retinal lesion with a pale center was noted which subsequently proved to be a local Candida abcess.

History.—This 53-year-old housewife was admitted Jan. 28, 1955, to the National Cancer Institute under the care of Dr. Donald Watkin, with a complaint of 18 months of increasing fatigability and 15 months of pain in the left shoulder and arm. Twelve months before admission multiple bone lesions had been found by x-ray, biopsy confirmed a diagnosis of multiple myeloma, and some symptomatic relief was obtained with urethan treatment. Nine months prior to admission Bence Jones proteinuria and anemia appeared along with increasing bone pain.

System review was negative aside from an episode of hemoptysis two months before admission and some shortness of breath. She had complained of pruritus ani.

In February, C. albicans was cultured from a vaginal swab. On March 26, 1955, a mild enteritis developed for which the origin could not be ascertained; no response was obtained from a short course of neomycin sulfate, and the loose stools persisted some four weeks. After the completion of balance studies, prednisolone (Meticortin), 100 mg. daily for 8 days, was started May 8, 1955, then 1000 mg. daily for 10½ days, which provided some relief of bone pain but had to be withdrawn in the latter part of the month owing to increasing mental changes and confusion. Because of a history of recent head injury, a ventriculogram was done to rule out a subdural hematoma. By this time her hemoglobin value had fallen to 8.9 gm. and she suffered a bout of pneumonitis, which responded to penicillin, 2,000,000 units daily; streptomycin, 2 gm. daily, and erythromycin, 2 gm. daily, which were continued for two weeks. In early June she developed ventricular flutter, which responded to quinidine, and suffered symptoms compatible with hepatic encephalography, which was confirmed by sulfobromophthalein (Bromsulphalein) retention of 29% and arterial ammonium level of 3μg. per cubic centimeter. On June 6, 1955, C. albicans was cultured from the urine, and this infection was subsequently confirmed 12 times. C. albicans was cultured from the blood on June 14, 1955, and isolation of the organism was obtained from 10 later samples. At this time all antibiotics were discontinued. Cultures of C. albicans were also obtained from the throat and bone marrow.

On June 15, 1955, a retinal lesion was noted in the left eye. This lay above the disk about a disk diameter from its margin and consisted in a rounded hemorrhagic area, the center of which was white. The lesion was apparently uninfluenced by the pattern of the nerve fiber layer and was not in relationship to a blood vessel. In the next few days this enlarged by inferior and lateral extention to appear as shown in Figure 1. At this time Dr. William Mohler correctly suggested that this might be a local C. albicans colony in the retina. Therapy with nystatin (Mycostatin) was cautiously attempted, but the patient proved intolerant to the medication.

The patient's subsequent course was complicated by femoral thrombophlebitis and continued pneumonitis, and death occurred following acute substernal pain on July 15, 1955.

Fig. 1.—The appearance of the retinal Candida abscess at ophthalmoscopic examination.

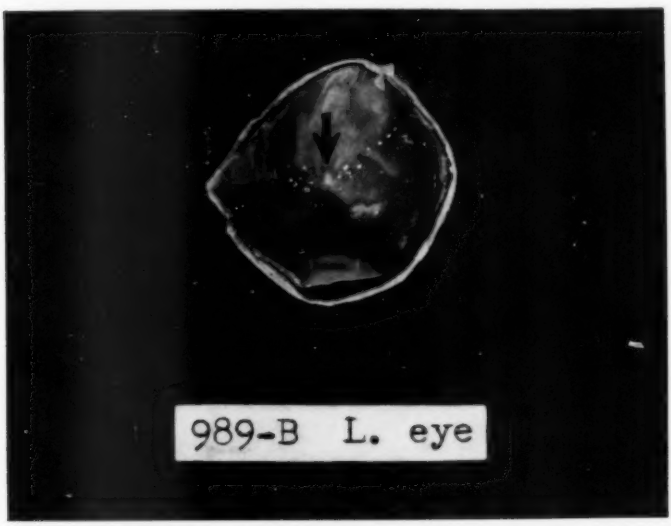

Fig. 2.—The appearance of the retinal Candida abscess which lies directly above the optic disk after fixation in 10% formalin. The elongated folded white area to the right of the disk is autolytic swelling of the perimacular area. The arrow indicates the lesion.

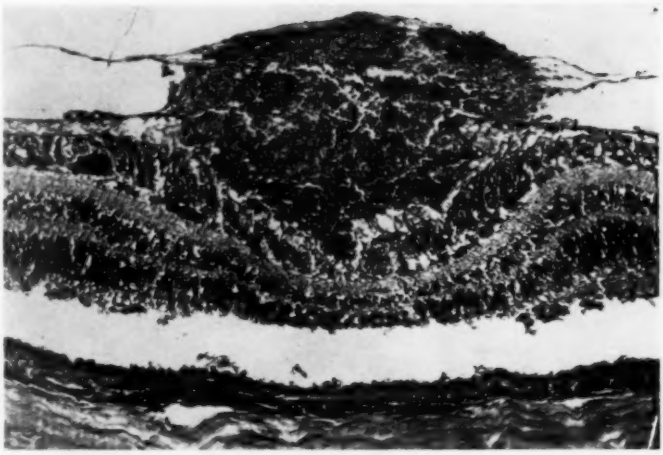

Fig. 3.—The major Candida abscess, which appears to originate in the nerve fiber layer, depresses and thins the subjacent retinal layers and has burst through the internal limiting membrane. The hyaloid membrane has separated from the internal limiting membrane, and exudate is spreading laterally in the subhyaloid space from the major mass of the abscess. Hematoxylin and eosin; reduced 35% from mag. × 150.

Pathological Examination.—General postmortem examination disclosed widespread multiple myeloma involving the calvarium, vertebrae, clavicle, sternum, ribs, and femora, with a Bence Jones nephrosis. C. albicans granulomata were found in the lung, heart, kidney, liver, and brain. Thrombosis was present in the right iliac vein, and emboli were found in the smaller pulmonary arteries.

At postmortem examination, five hours after death, the posterior halves of the eyes were removed and fixed in 10% formalin. Although it is our custom to preserve the retina by injection of the vitreal cavity with formalin immediately after death, this was unfortunately not carried out in the present instance. As a result, the retina showed postmortem autolytic defects, such as fragmentation of the outer segments of the bacillary layer and swelling of the retina in the perimacular region.

After fixation, the retinae showed a white area (Fig. 2) above the disk. The eyes were imbedded in paraffin and serially sectioned. Sections were cut at 5μ and retained at millimeter intervals in the periphery and at 500μ intervals in the region of the papilla. When the larger retinal lesion was seen, extra sections were taken at this level. Sections at each level were stained with hematoxylin and eosin, Gomori's trichrome method, and the periodic acid-Schiff (PAS) reagent stain with hematoxylin counterstain.

The major retinal lesion had evidently taken origin in the nerve-fiber layer (Fig. 3). It displaced and thinned the subjacent ganglion-cell layer and more peripheral layers, although it did not invade them. It penetrated the internal limiting membrane and spread out over the retinal surface in the subhyaloid space (Fig. 4). It is a commentary on the integrity of the hyaloid membrane that it so completely contained the exudate.

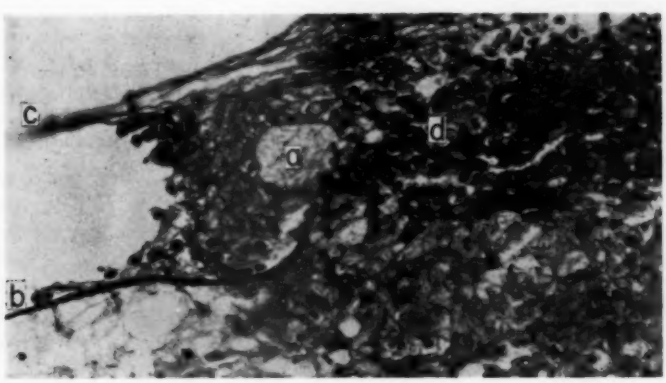

Fig. 4.—The lateral margin of the abscess. (*a*) One of numerous clear bodies with distinct margins and lacking a nucleus (? "cytoid body"). (*b*) Internal limiting membrane. (*c*) Hyaloid membrane. (*d*) Yeast elements. Periodic acid-Schiff reagent and hematoxylin; reduced 37% from mag. × 500.

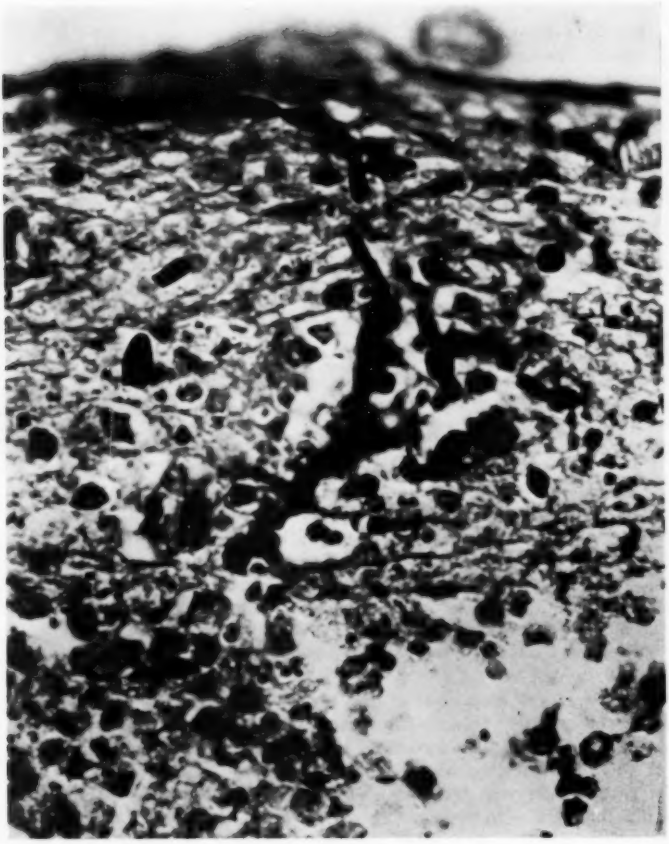

Fig. 5.—C. albicans in the retinal abscess in hyphal and blastospore form. Periodic acid-Schiff reagent and hematoxylin; reduced 10% from mag. × 1050.

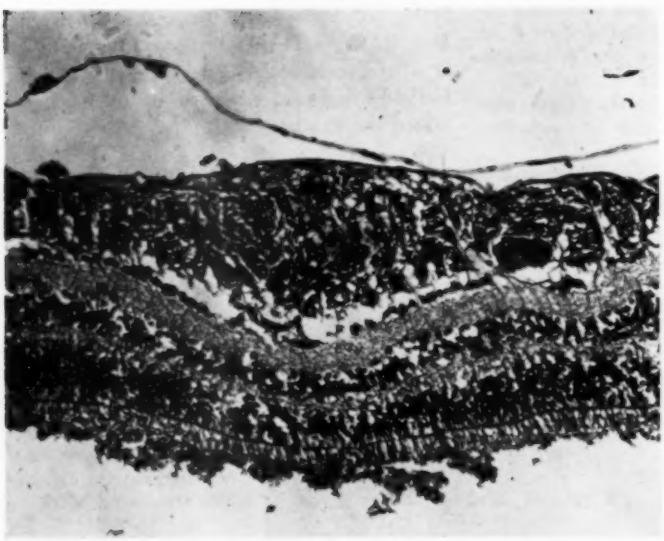

Fig. 6.—A second smaller granuloma nearer the optic disk in which no yeast elements were found. Hematoxylin and eosin; reduced 13% from mag. × 225.

Fig. 7.—Mononuclear and polymorphonuclear elements overlying the optic disk in the subhyaloid space. Hematoxylin and eosin; reduced approximately 40% from mag. × 80.

The nerve-fiber layer lateral to the mass appeared as though distended by fluid. The granuloma itself contained the yeast both in hypal and in budding form, though there were no dense aggregates (Fig. 5). Although numbers of recognizable inflammatory cells were present (both polymorphonuclear and mononuclear cells), the larger part of the granuloma was made up of nuclear debris and rather amorphous material. Some of these were bodies with an evident membrane but contained no nucleus or definite intracellular structures (Fig. 4*a*). Although these may be called cytoid bodies, I prefer to limit the use of this term to the varicose degeneration of nerve fibers (i. e., as in papilledema). Although these might have been degenerative nerve fibers, I had no proof of it.

Where the lesion had spread in the subhyaloid space but the internal limiting membrane remained intact the subjacent nerve fiber layer was widened by distention of the tissue spaces as though by fluid. Just lateral to the disk a much smaller granuloma was found (Fig. 6). No yeast elements were present on the PAS stain, and only rare mononuclear cells which could be interpreted as inflammatory cells were present. The bulk of this lesion was made up of the amorphous vacuolated debris mentioned above. In addition, well-stained elongated nuclei of irregular outline and with sparse chromatin, sometimes as long as 15μ, were seen. The nature of these cells suggests that they were of mesodermal origin.

One small lesion was seen in the choroid. This consisted in some thickening of the layer and a moderately dense accumulation of mononuclear inflammatory cells, but no yeast bodies could be found on adjacent sections stained with PAS.

Inflammatory cells were present in the subhyaloid space and in particular had wandered over the optic papilla. These cells were both polymorphonuclear and mononuclear and were in the form of small aggregates (Fig. 7).

Comment

In 1872, Roth[38] described round white retinal spots which he ascribed to varicose degeneration in the nerve fiber layer. In the same year[39] he associated these retinal lesions with a great variety of constitutional disease. In one, at least (his Case 5), the patient had been found to suffer from a mixed septic and embolic process, apparently an acute bacterial endocarditis.

Since this time, the retinal lesions associated with systemic septic disease have been called "Roth spots," although by their common definition they are different from what this author described. Duke-Elder,[40] 1941, defined the lesions as round or oval spots in the retina, usually near the disk and often associated with hemorrhages.

The retinal lesions, or "Roth spots," associated with a systemic septic process have been best described in bacterial endocarditis. The most complete recent reports are by

Doherty and Trubeck,[41] 1931, and Dienst and Gartner,[42] 1944. The lesions were found to have hemorrhagic margins and might or might not have white centers. They lay near the disk, usually not further than 2.5 disk diameters from the disk margin, and did not involve the macular area. On histological examination in a case of subacute bacterial endocarditis, Doherty and Trubeck,[41] 1931, found nodular swellings of the nerve-fiber layer lacking cellular infiltrate. The lesion was homogeneous and resembled coagulated albuminous material. In an instance of acute bacterial endocarditis they found lesions of two types. Although both were localized in the nerve-fiber and ganglion-cell layers, one consisted of an area of free and phagocytosed blood pigment with edema of the subjacent nerve-fiber layer but was practically free of inflammatory cells. The second type consisted in a mass of inflammatory cells containing polymorphonuclear leukocytes, which passed through the total retinal thickness and terminated at the pigment layer. Dienst and Gartner,[42] 1944, likewise saw the lesions originating in the nerve-fiber layer, some of which contained many polymorphonuclear leukocytes and appeared as small abscesses. In others, however, the inflammatory response was considerably less active. In these (which were noted to be the majority), the cellular response consisted in small round cells, "tissue cells" (not further defined), plasma, epithelioid cells, and occasional giant cells. Dellmann,[43] 1919, presented a thoughtful study of a patient with subacute bacterial endocarditis in whom gross retinal lesions were found consisting in hemorrhages and small yellow-gray nodules resembling tubercles. The most interesting feature of this case was the finding of a thick plug of cocci in a capillary near the central vessels of the retina which appeared to totally lack a surrounding inflammatory response. In summing up the evidence in the present case, as well as that in the literature, there appear to be two extremes of the retinal lesions. On one hand the lesion may be frankly inflammatory and on the other it calls forth a varying quantity of elements, which appear to be nonmyeloid in nature and which apparently others, in addition to myself, have had some difficulty in identifying.

Friedenwald, 1952, considers the white spots as small infarcts in the nerve fiber layer, due to the blockage of terminal arterioles by septic emboli. Dellmann's case provides proof that even obvious bacteria may, under proper conditions, not excite an inflammatory response in the retina and thus act simply as occlusive emboli. It seems hard, however, to credit this hypothesis, since the lesions, when small, are so precisely localized in the nerve-fiber layer with sparing of the subjacent layers which would seem dependent upon the same blood supply. It is curious, too, that if the lesions are due to microscopic infarcts some might not occur in the capillary network of the internal granular layer and provide a lesion, clearly originating here rather than in the relatively avascular nerve-fiber layer, inasmuch as hemorrhages from other causes (Friedenwald, 1952) do occur in the internal granular layer. Such a case has not come to my attention.

The suggestion that this is a varicose degeneration of nerve fibers is also not completely acceptable, since there seems no evidence of this degeneration appearing along the full length of the axon as might be reasonably expected. On the contrary, both ophthalmoscopically and histologically, these are discrete lesions, suggesting a local exudation or change, possibly in the supporting rather than the conducting tissue of the retina. A study of the reactions of glial framework of the retina (Müller's fibers) would be of greatest interest in other instances of these lesions. One may speculate whether swelling and degeneration of the foot plates of these glial fibers at their insertion on (or indeed formation of) the internal limiting membrane might not account for the histological picture seen by the incomplete chromatic methods. It

is proper, however, to note that no evidence of a similar reaction has been found to be present at the peripheral termination of these cells (at the external limiting membrane), nor is it evident in any of the illustrations available to me.

Summary

A case of septic retinitis due to Candida albicans is presented, and the relationship of this yeast to systemic infection is outlined. The lesions appear to originate in the nerve-fiber layer, and hyphal and budding forms of the yeast were demonstrated in one lesion. An additional type of retinal lesion was seen in which there was little, if any, evidence of inflammatory reaction. Two types of retinal lesions have been found, both in the present case and in previous cases of bacillary septicemia and embolism. Both arise in the nerve-fiber layer. The reason for the apparently exclusive origin in the nerve-fiber layer of both types of lesions is not clear. One type of lesion is frankly inflammatory and seems adequately explained as a result of a septic embolus. The second type, perhaps more properly termed a Roth spot, is a more localized lesion, the response to which is predominantly noninflammatory. Difficulties are pointed out in accepting the latter lesion as being due to embolism, infarction, or varicose axonal degeneration of the retinal ganglion cells.

The attending physician, Dr. Donald M. Watkin; the clinical associate, Dr. William C. Mohler, of the Nutrition and Metabolism Service, General Medicine Branch, National Cancer Institute, and Dr. Louis B. Thomas, Head of Surgical and Post Mortem Services, Pathologic Anatomy Branch, Clinical Center, gave permission for study of this case.

Branch of Surgical Neurology, National Institute of Neurological Diseases and Blindness (14).

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Injury to Liver Cells in Carbon Tetrachloride Poisoning

Histochemical Changes Induced by Carbon Tetrachloride in Mouse Liver Protected by Sulfaguanidine

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Introduction

Carbon tetrachloride (CCl_4) has been used extensively as a hepatotoxic agent in the study of regeneration after acute injury and in the study of fibrosis and tumor development after chronic poisoning. A single administration of CCl_4 induces necrosis of the centrolobular cells of the liver within 24 hours, followed by rapid proliferation of surviving cells in the portal half of the lobule until the original mass and cytoarchitecture of the liver are restored, within 5 to 6 days.[1-7] In the course of studies of mitosis and other nuclear phenomena in the mouse liver it was found that in mice supplied with various sulfonamide compounds the livers exhibited varying degrees of protection from CCl_4 injury.[8,9] When administered at sufficiently high levels, sulfaguanidine inhibits completely the necrosis of the centrolobular cells of the liver, although transient changes in these cells do occur. It was hoped, therefore, that a comparison of livers protected by sulfaguanidine with unprotected livers might provide some information about the mode of action of CCl_4.

This is a report on some cytological and histochemical changes in such livers during the first 48 hours after CCl_4 was administered. The same changes in cytoplasmic ribonucleoprotein, glycogen, and neutral fat occur in protected and unprotected livers during the first 18 hours, but alterations in the mitochondria and, subsequently, in the nuclear deoxyribonucleoprotein occur only in unprotected livers and are indications of irreversible damage.

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Material and Methods

Young adult mice from two inbred strains, BUB and a subline of C3H, maintained in this laboratory were used. They were 2 to 4 months old and weighed 28 to 32 gm. The animals were raised on Purina Laboratory Chow in rooms maintained at 78 F.

Three days before administration of CCl_4 the mice were given basal semisynthetic diets ad libitum. One-half of the mice received the basal diet, containing 30% casein, 47% corn starch, 8% yeast powder, 7% salt mixture, 4% hydrogenated vegetable fat, 2% corn oil, 2% cod liver oil.* The remaining animals received a similar diet, with the exception that 2% of the corn starch was replaced by sulfaguanidine.† In preliminary experiments it was found that three days' feeding on the latter diet is sufficient to assure protection from CCl_4 injury.

Carbon tetrachloride was administered by subcutaneous injection of 0.1 ml. of a 40% solution of the CCl_4 in sesame oil. This method was chosen because of its simplicity and because uniform in-

* Diet ingredients: Sodium caseinate, Nutritional Biochemicals Corp.; Salt mixture No. 2, U. S. P. XII, and Brewer's Yeast Powder, General Biochemicals, Inc.; Argo Corn Starch; Crisco; Mazola corn oil; Squibb Cod Liver Oil.

† Sulfaguanidine was supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

jury is obtained with it. Varying doses from one-half to twice the amount used here give the same degree of damage; that is, approximately one-third to one-half of the liver lobule is necrotic 24 hours after injection. None of the mice died following this treatment. The injection was always made between 8:30 and 10:30 a. m. to avoid any possible changes due to differences in the time of day when the injection was made.

Three to six mice were killed at each of the following times after injection: 15, 30, and 45 minutes and 1, 2, 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, and 48 hours. They were stunned, decapitated, and bled thoroughly, and the livers were fixed immediately.

Routine histological changes were observed in tissues fixed in Bouin's fluid and stained with Delafield's hematoxylin and eosin. Deoxyribonucleic acid was revealed by the Feulgen method[10] after fixation in Serra's fluid. Ribonucleic acid was demonstrated as the basophilic component of the cells that was digestible by ribonuclease. The tissues were fixed in Serra's fluid for not more than one hour[11] and according to Singer's method[12] were stained in 5×10^{-4} molar methylene blue, pH 5.3, at 25 C for three hours and dehydrated through tertiary butyl alcohol. Before staining, control sections were incubated 30 minutes at 37 C in a 0.1% solution of ribonuclease at pH 6.7. Glycogen and other polysaccharides were stained by the periodic acid-Schiff technique[13,14] in tissues fixed in ice cold Rossman's fluid. Neutral fat was preserved by fixation in 10% buffered formalin, pH 6.8, and stained in frozen sections with Sudan IV or Sudan black B. Mitochondria were fixed and stained by the Regaud method.[15] Succinic dehydrogenase activity was determined in sections by Padykula's modification[16] of the Seligman and Rutenburg method.

Observations

Ribonucleoprotein.—After CCl_4 administration one of the first changes that is demonstrable histochemically is a dispersal and apparent diminution in amount of the basophilic component of the cytoplasm in the centrolobular cells. This substance is digestible with ribonuclease and thus represents the ribonucleic acid-containing fraction of the cytoplasm. In centrolobular cells of control livers it is aggregated into relatively large irregular masses, the so-called "basophilic bodies" (Fig. 1). Dispersal of these aggregates is detectable as early as 15 to 30 minutes after injection of CCl_4, at a time when the cells begin to appear swollen, and is complete after 2 hours. Subsequently, until 15 to 18 hours after injection, a diffuse faint basophilia persists (Fig. 2). This sequence is found both in unprotected livers and in those protected by sulfaguanidine. After 18 hours, however, all traces of stainable ribonucleoprotein are lost from the centrolobular cells of unprotected livers (Fig. 3), whereas a restoration of basophilic bodies begins in the protected livers (Fig. 4). During these alterations in the centrolobular cells, the cells in the portal zone of the lobule undergo no change in ribonucleoprotein distribution that is detectable by light microscopy.

Glycogen.—Concomitant with ribonucleoprotein dispersal there is a gradual diminution and eventual loss of glycogen in livers which are not protected by sulfaguanidine. At the time of day selected for administering the CCl_4, all cells of the hepatic lobule are heavily laden with glycogen. Diminution of glycogen in the central zone of the lobule is discernible 15 to 30 minutes after injection; after 1½ to 3 hours a sharp line of demarcation between the swollen glycogen-poor central cells and the glycogen-rich periportal cells is established; after 5 to 6 hours the central cells are devoid of glycogen, and they remain so thereafter (Fig. 9). There is also a transient loss from the periportal cells which begins approximately 6 hours after injection and is complete after 12 to 15 hours. In the normal diurnal cycle glycogen would be abundant at these times. Restoration of glycogen in these surviving portal cells begins only at 30 hours and is complete at 48 hours.

In the livers protected by sulfaguanidine there is a transient loss of glycogen from the centrolobular cells which is first detectable three hours after injection and is complete after six hours. Thereafter, glycogen returns gradually to this zone. Glycogen also is affected in the portal cells of the livers protected by sulfaguanidine in that, although it remains normally high through the first 6 hours and then decreases normally

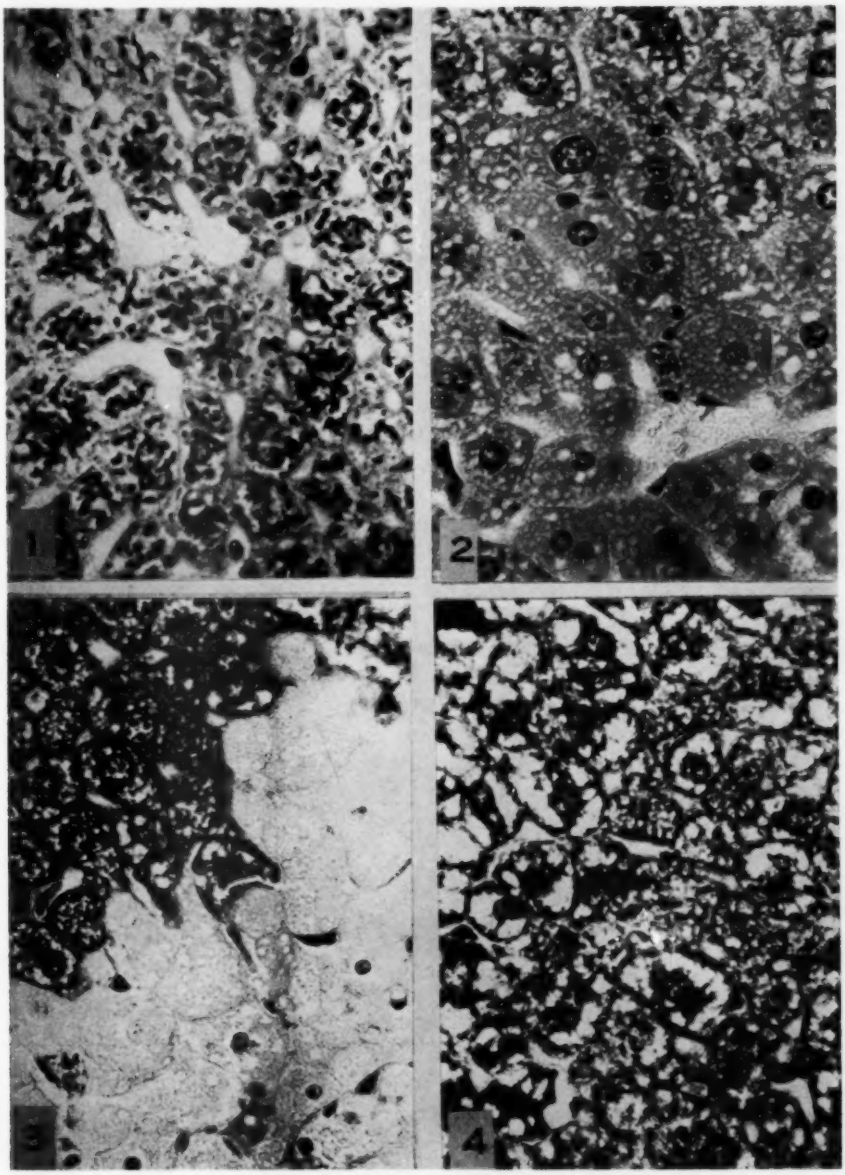

Plate 1.—Mouse liver fixed in Serra's fluid and stained with methylene blue. In Figures 2 to 4 periportal cells are in upper left and centrolobular cells, in lower right of photomicrograph; × 7.5 ocular, × 60 objective, bellows length 26 in.

Fig. 1.—Basophilic bodies in cytoplasm of centrolobular hepatic cells of a control mouse maintained on the basal diet but not treated with CCl_4.

Fig. 2.—Dispersal of basophilic bodies so that only diffuse basophilia persists in centrolobular cells in a mouse protected by sulfaguanidine, six hours after CCl_4 injection.

Fig. 3.—Disappearance of all basophilic substance from cytoplasm of centrolobular cells in an unprotected mouse, 24 hours after CCl_4 injection.

Fig. 4.—Reappearance of small basophilic bodies in centrolobular cells, 24 hours after CCl_4 injection, in a mouse protected by sulfaguanidine.

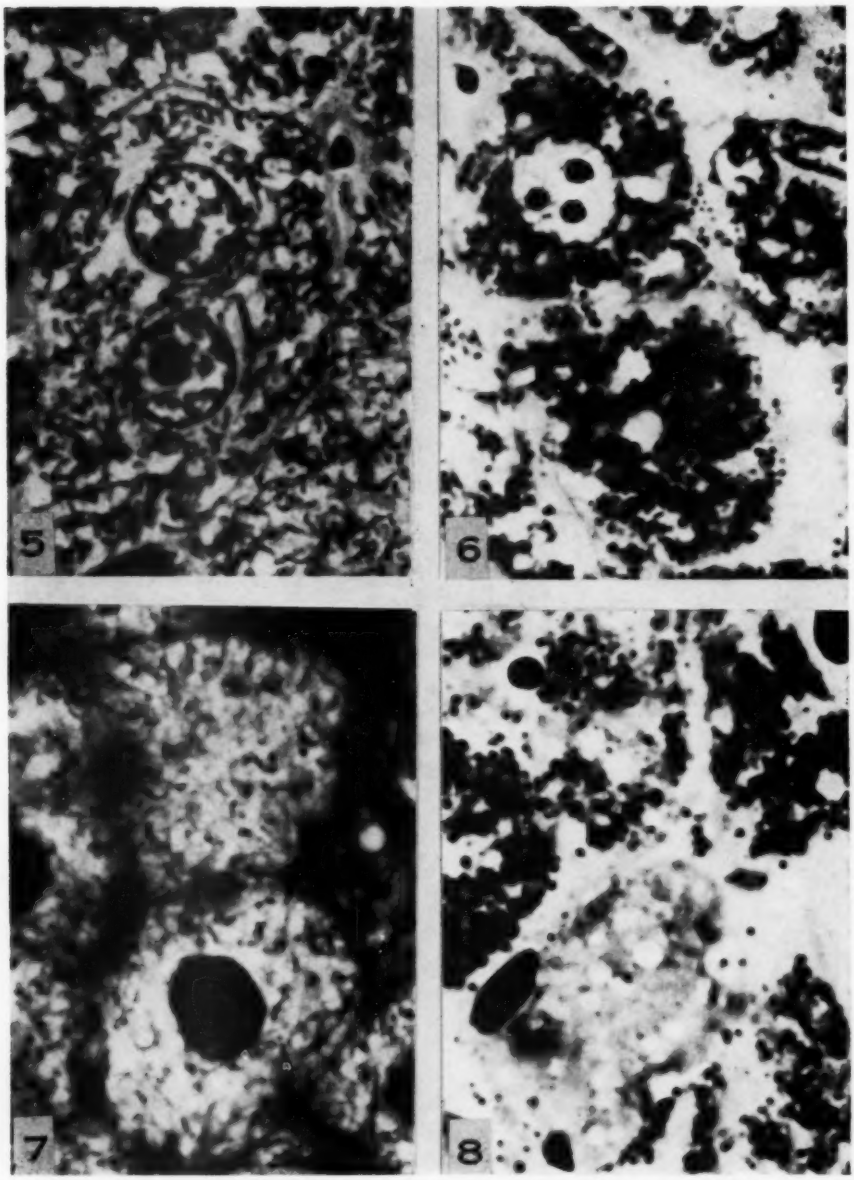

Plate 2.—Mouse liver fixed and stained by Regaud's techniques; × 12.5 ocular, × 90 objective, bellows length 33 in.

Fig. 5.—Normal filamentous mitochondria in centrolobular hepatic cells of a mouse protected by sulfaguanidine, six hours after CCl_4 injection.

Fig. 6.—Enspherulated mitochondria in centrolobular cells of an unprotected mouse, six hours after CCl_4 injection.

Fig. 7.—Filamentous mitochondria in cells distended by hydropic vacuoles in liver of a mouse protected by sulfaguanidine, 15 hours after CCl_4 injection.

Fig. 8.—Enspherulated mitochondria which vary in size, in number per cell, and in degree of staining, 21 hours after CCl_4 injection, in centrolobular cells of an unprotected mouse.

9 hours after injection, it remains low until 24 hours after injection during a period in which the liver is usually heavily laden with glycogen. Hence, transient changes in glycogen storage in the periportal cells occur in both protected and unprotected livers, but the effect is of shorter duration in the former.

Neutral Fat.—As the glycogen content of the centrolobular cells wanes, neutral fat stainable with sudan dyes accumulates. At the time of injection there are only trace amounts of demonstrable fat in the centrolobulbar cells and usually none in the periportal cells. Lipid droplets begin to accumulate in the centrolobular cells of unprotected livers 9 hours after CCl_4 injection and are very abundant from 12 hours (Fig. 10) until the cells disappear. The periportal cells become similarly laden 3 to 6 hours later, but they lose this fat around 48 hours after CCl_4 injection. In livers protected by sulfaguanidine, the centrolobular cells exhibit the same increased deposition of fat 9 to 12 hours after injection, but there is a gradual return to the very small amounts characteristically found in untreated mice by 24 hours after injection. Fat in the periportal cells in these protected livers is present only in trace amounts, if any, at all times.

Mitochondria.—In the normal mouse liver, mitochondria exhibit varying forms in different parts of the lobule. There is a transition from long slender filaments in the centrolobular cells to short thick rods in the periportal cells.

In unprotected livers after CCl_4 administration an enspherulation of the mitochondria in the centrolobular cells begins two to four hours after injection. At six hours all mitochondria in this zone of the lobule have been transformed into small spheres (Fig. 6). From 12 through 36 hours (Fig. 8) some of these enspherulated mitochondria become swollen; their size may vary greatly from cell to cell or even within a single cell; their outlines become less distinct; they stain less readily; they vary considerably in number from cell to cell; they frequently tend to clump together, and a few isolated cells become devoid of mitochondria. The distribution of cells with few mitochondria as compared to those containing many is random in the prenecrotic zone. Although in other respects the cells appear necrotic from 21 hours after injection, the mitochondria remain abundant and relatively well defined (Fig. 8) through 36 hours. After 48 hours they stain very faintly or not at all, but their outlines are still discernible in most cells. The mitochondria of the periportal cells, on the other hand, are unaffected and retain their normal appearance.

In the livers of animals protected by sulfaguanidine, the mitochondria retain their normal filamentous form in the centrolobular cells (Fig. 5), even in occasional livers in which hydropic vacuolization develops (Fig. 7).

Succinic Dehydrogenase Activity.—This was studied in an attempt to correlate changes in activity with changes in mitochondrial form. In the livers of untreated mice activity is greatest in the periportal cells and least in the centrolobular cells. This activity persists in all the animals protected from CCl_4 damage by sulfaguanidine and also in the unprotected mice through approximately 30 hours. After 36, 48, and even 72 hours, activity persists in some cells but disappears from others in the necrotic zone.

Deoxyribonucleic Acid.—The deoxyribonucleic acid (DNA) of the nuclei of the parenchymal cells is not visibly affected during the first nine hours after CCl_4 administration (Fig. 11). After 12 to 15 hours a variable number of nuclei in the unprotected livers begin to undergo changes, and after 18 to 21 hours all centrolobular-cell nuclei are affected. Four types of changes occur. Most of the nuclei become pycnotic (Fig. 12); others become karyorrhetic, then fragment into several small Feulgen-positive spheres (Fig. 14); some appear to shrink slowly and gradually lose their capacity to stain (Fig. 13) and, there-

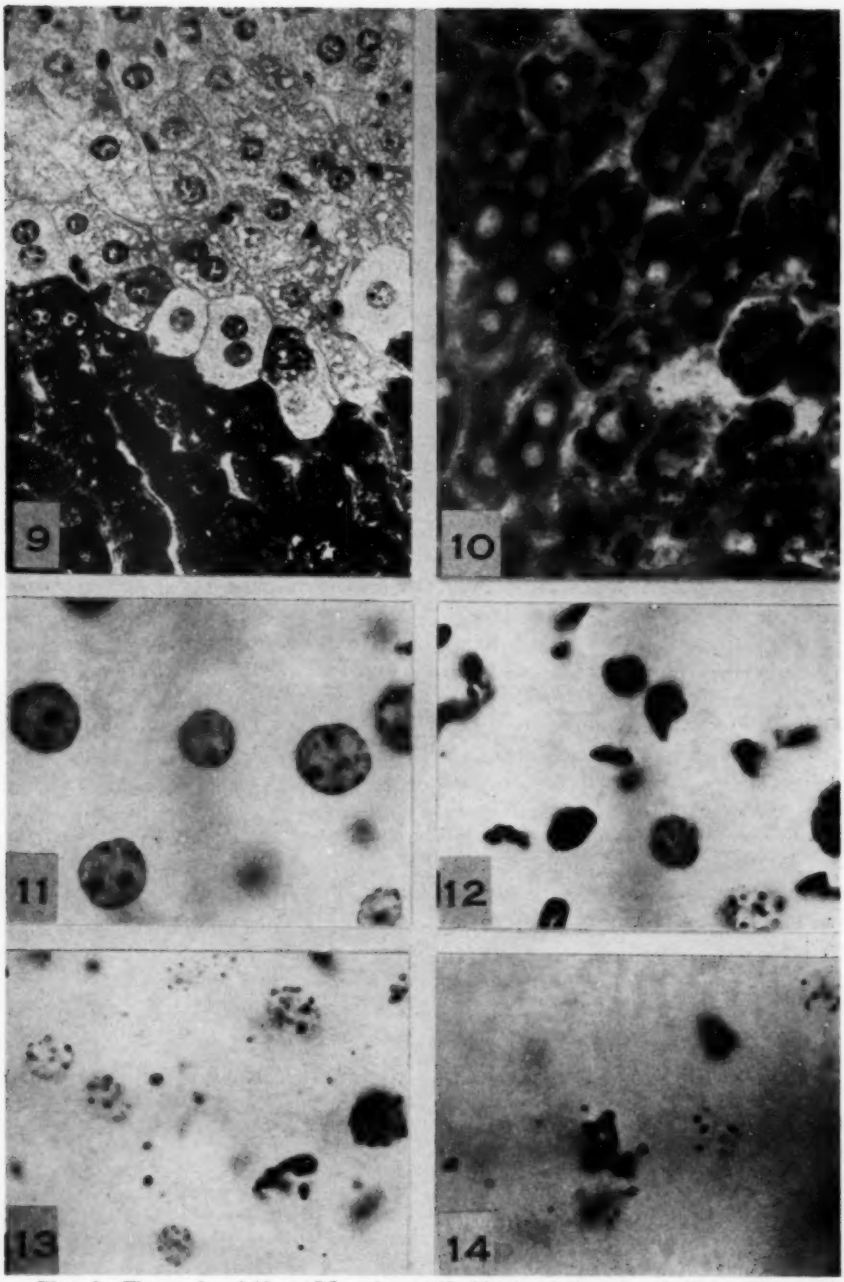

Plate 3.—Figures 9 and 10: × 7.5 ocular, × 60 objective, bellows length 26 in. Figures 11 through 14: × 12.5 ocular, × 90 objective, bellows length 26 in.

Fig. 9.—Loss of glycogen from centrolobular cells (upper half of photograph) of an unprotected mouse, six hours after injection of CCl_4. Periportal cells (lower half of photograph) heavily laden with glycogen (periodic acid-Schiff technique).

Fig. 10.—Accumulation of droplets of sudanophilic lipids in centrolobular cells (upper and right halves of photograph) of an unprotected mouse, 12 hours after injection of CCl_4 (Sudan black B).

Fig. 11.—Normal nuclei in centrolobular cells of an unprotected mouse, nine hours after CCl_4 injection (Feulgen).

Fig. 12.—Pyknotic nuclei in centrolobular cells of an unprotected mouse, 15 hours after CCl_4 injection (Feulgen).

Figs. 13 and 14.—Loss of DNA and fragmentation of nuclei in centrolobular cells of an unprotected mouse, 21 hours after CCl_4 injection (Feulgen).

fore, their content of DNA; a few nuclei appear to lose their turgor and collapse. After 24 hours most cells undergoing necrosis still contain traces of DNA; after 30 to 36 hours most of the DNA has disappeared, and its dissolution is complete after 48 hours. During this period the periportal nuclei are not affected. In livers protected by sulfaguanidine the nuclei throughout the lobule are unaffected.

Hydropic Vacuoles.—Twenty-four hours after CCl_4 administration in the rat, Cameron and Karunaratne [2] described enlarged "balloon-like" cells distended with hydropic vacuoles, situated at the margin between the necrotic centrolobular cells and the periportal surviving cells. In the livers of our unprotected mice a small number of presumptive balloon cells appeared 6 hours after CCl_4 injection and persisted through 18 to 21 hours. No discrete hydropic vacuoles could be detected in the damaged centrolobular cells, but a swelling of the cells was apparent as early as 15 to 30 minutes after injection, which suggested an imbibition of fluid.

The extent of protection by sulfaguanidine may vary. In some cases it is so complete that with ordinary histological methods no injury whatever is demonstrable. On the other hand, although the centrolobular cells of livers protected by sulfaguanidine do not undergo necrosis, they occasionally and very sporadically undergo a form of "hydropic vacuolization" which differs from that of balloon cells. The affected cells become swollen and filled with so many small vacuoles that the cytoplasm between them is compressed into extremely thin partitions, and there often is found a clear blister-like vacuole indenting one side of the nucleus. Glycogen is very sparse in such cells, limited to occasional deposits in the thin cytoplasmic partitions between the vacuoles, and fat is only variably present. This vacuolization has been found in animals killed after various intervals from 6 to 48 hours after CCl_4 injection. The number of such cells is very variable; there may be only a single row of cells immediately around the central vein, or the entire central zone of the lobule may undergo a vacuolization of the cytoplasm of the parenchymal cells. We regard this as a form of incipient cell injury which is reversible, because we never find necrosis in animals killed after later intervals.

Necrotic Changes.—In the centrolobular cells of unprotected livers there is a transient appearance of discrete but irregular and amorphous masses which may represent sites of early cytoplasmic destruction. They are well defined from 6 through 12 hours after injection but subsequently appear to merge with the rest of the cell constituents. They exhibit no unique staining characteristics. The cytoplasm of the cells that are becoming necrotic undergoes two other changes which are first discernible at 18 hours and distinct at 24 hours. The cytoplasm becomes progressively more eosinophilic and more stainable by the periodic acid-Schiff method. These changes are discussed by Williams.[5] Nothing like this is found in the sulfaguanidine-protected livers.

Comment

Investigators generally agree that an early effect of CCl_4 is swelling of the centrolobular hepatic cells, apparently by imbibition of fluid.[4,7,17,18] The puzzling fact that this swelling plus subsequent necrosis occurs in the central zone of the lobule, instead of the periportal zone where the cells are first exposed to the circulating poison, has been interpreted by Glynn and Himsworth [19] as a secondary and indirect action of the CCl_4. They suggest that a swelling of the hepatic cells produces an ischemia of the organ, which in turn brings about a deficiency in the oxygen supply of the centrolobular cells, and that necrosis is the result of this anoxia. Myren,[7] on the other hand, proposes that hepatic cells, especially in the midzone, are directly attacked by CCl_4; then the swelling of these cells impedes circulation, and the injury to centrolobular cells follows. The involvement of anoxia in CCl_4-induced necrosis has been questioned, however, be-

cause there is some evidence that there is no impairment of circulation of blood in the liver after CCl_4 administration.[20] Recent investigations have emphasized a possible direct effect of CCl_4 which brings about a disruption of the enzyme systems of the cell,[18] but there is no explanation for the selective effect on those cells in the central zone of the hepatic lobule.

There have been several reports on the early histochemical changes in the livers of unprotected mice after CCl_4 poisoning,[4-7,21] and our observations on ribonucleic acid, glycogen, neutral fat, deoxyribonucleic acid, mitochondria, and succinic dehydrogenase activity are in general agreement with those of other investigators. Differences in the times at which these changes occur are probably attributable to differences in doses and routes of administration of CCl_4; the species, strain, and age of animals, and possibly the time of day of the injection, because a diurnal rhythm exists in the liver, even in fasting animals. This study was carried out for the purpose of comparing the changes in unprotected livers with those in livers protected by sulfaguanidine. Although no necrosis occurred in the latter after CCl_4 injection, the same dispersal of cytoplasmic basophilic bodies, diminution of glycogen, and accumulation of neutral fat were observed. These three phenomena, therefore, are reversible and do not lead to cell death.

The initial loss of the aggregates or clumps of basophilic material from the centrolobular cells was interpreted at first as a diminution in amount of ribonucleoprotein.[4,5,22] Quantitative analyses,[17,18,21,23] however, although difficult to interpret, since both damaged and undamaged cells are included in the aliquots used for chemical determinations, have not supported this idea. Hence there may be simply a dilution effect[17] brought about by the entrance of water into the cells, with a resulting dispersal of the aggregates. The cytoplasmic ribonucleoprotein complex as revealed by the electron microscope is normally organized, in part, as a system of interconnected flattened, membrane-bounded vesicles, the endoplasmic reticulum,[24,25] and it seems likely that the permeability of this membrane as well as those of cell membranes and mitochondrial membranes[18] may be increased by CCl_4. A swelling of the vesicles by penetration of fluid through their membranes could account for the dispersal of the basophilic bodies that are discernible with the light microscope without an actual loss of substance from the cell. That such a dilatation of the endoplasmic reticulum does occur after acute CCl_4 poisoning has been demonstrated recently by Oberling and Rouiller[26] by electron microscopy. These authors assert, furthermore, that the hydropic vacuolization which occurred in some cells in their experiments was largely the result of this swelling. On the basis of these considerations our observations of the similar but transient early changes in the basophilic bodies plus the occasional development of hydropic vacuoles in the "protected" livers indicate that sulfaguanidine, although it prevents necrosis, does not prevent CCl_4 from affecting cell membranes and the membranes of the endoplasmic reticulum.

The loss of glycogen and accumulation of fat in the unprotected liver can be interpreted as expressions of disturbed metabolic activity. The significance of these changes is not known; but, for example, the early loss of glycogen from the prenecrotic cells might result from anaerobic glycolysis, and the subsequent loss from the surviving cells, from a drain on liver reserves for blood-sugar maintenance and for energy requirements for growth. The concomitant accumulation of sudanophilic lipids in the surviving cells may represent a temporary overload of the mechanism for lipid metabolism or a mobilization from depots in order to ensure a ready source of energy. The fact that glycogen depletion and excess fat accumulation also occur in the centrolobular zone of "protected" livers suggests that there actually is an incipient injury to

these cells which is sufficient to bring about a temporary metabolic disturbance but not great enough to cause cell death.

The mitochondria of the prenecrotic centrolobular cells in unprotected livers are transformed into swollen spheres a few hours after CCl_4 administration. It should be emphasized that enspherulation and swelling of mitochondria occur in response to a great variety of types of cell injury, and these changes are characteristic of the pathological state called "cloudy swelling."[27,28] This swelling is probably the result of imbibition of fluid, which could result either from an increase in the permeability of the mitochondrial membranes[18] or from a change which makes the matrix within the mitochondria more hydrophilic. They remain in this swollen state for 48 hours or more, although their ability to bind conventional dyes may be lost.[27] Histochemically demonstrable succinic dehydrogenase activity, which is associated with mitochondria, can be detected as long as 72 hours after CCl_4 injury, and complete loss of activity in a few isolated cells is first seen only at 36 hours. This persistence of succinic dehydrogenase activity in injured centrolobular cells has been observed in other laboratories.[18,29] The elegant experiments of Christie and Judah[18] indicate that CCl_4 damage does not produce an inhibition of specific enzymes but a disorganization in the topographical relationships of the enzymes, which results in a loss of their integrated activity. These investigators propose, therefore, that CCl_4 acts directly on the cell by increasing the permeability of the mitochondria; this, in turn, brings about a loss of enzymic co-factors and a consequent disruption of organized enzymatic control of the respiratory, synthetic, and energy-producing activities of the cell, changes which bring about death of the cell.

The mitochondria and their constituent enzymes, therefore, seem to be important targets for the injurious effect of CCl_4. In addition to mitochondrial changes there is a dissolution of nuclear deoxyribonucleic acid in unprotected livers which does not take place in protected livers. The breakdown of the nucleus, since it takes place after mitochondrial damage has occurred, probably is the result of disturbed respiratory activity in the cell. The implication in our experiments, therefore, as well as in those of Christie and Judah[18] and Frunder, Fischer, and Börnig,[30] is that the primary damaging effect of CCl_4 which is irreversible is on the mitochondria. Further evidence for this concept is the fact that the mitochondria in livers protected by sulfaguanidine retain their normal form. Even in cells that have been incompletely protected and that, therefore, have become distended with hydropic vacuoles, the mitochondria are not transformed into swollen spheres. According to this view, then, sulfaguanidine exerts its protective effect on the liver by inhibiting, in some way, the swelling of mitochondria and preventing the disruption of associated enzyme systems.

The exact mechanism by which sulfaguanidine acts is not known. Leach and Forbes[8] first reported the protective effects of sulfanilamide, sulfathiazole, and sulfapyridine. Wilson et al.[9] found that sulfaguanidine also was effective but that formosulfathiazole was not. The protective effect of sulfaguanidine is probably not related to its antibacterial effect on intestinal flora, to its antithyroid activity, or to acetylation mechanisms involved in its metabolism.[33] A variety of other compounds have been found to exert at least partial protection against CCl_4 injury,[22,31,32] and the mechanisms involved are also unknown.

One might conceive the action of CCl_4 on the unprotected liver as involving three progressive stages. The first would be the entrance of CCl_4 into the cells. The fact that only centrolobular cells are involved suggests that only these cells are functionally capable of admitting the injurious agent. The second step is manifested by the rapid (15 to 30 minutes) swelling of the cells and dispersal of their basophilic bodies, phenomena which can result from imbibition of

excess fluid. This may be brought about by changes in the permeability of cell membranes and membranes of the endoplasmic reticulum. Carbon tetrachloride is a lipid solvent, and it might well attack the lipid component of intracellular membranes. Finally, in the third stage, which occurs two to four or more hours after CCl_4 administration, the mitochondrial membranes are attacked, possibly in the same manner. In this hypothetical scheme sulfaguanidine exerts its protective effect at some point between the second and third stages. It is not entirely certain at that time, however, that CCl_4 attacks the mitochondria directly; there may be a still undetected series of events which lead to the disruption of mitochondria, and it is here that CCl_4, as well as other unrelated agents which produce centrolobular necrosis, may act. A possible alternative explanation, then, is that sulfaguanidine does not protect the liver against the direct action of CCl_4 but does protect against some consequencies of its action.

Summary

Histochemically detectable changes in mouse liver after subcutaneous injection of a single acute dose of carbon tetrachloride (CCl_4) have been studied during the development of centrolobular necrosis and compared to those in livers protected from this necrosis by sulfaguanidine.

In the centrolobular cells of both protected and unprotected livers CCl_4 administration induces a rapid dispersal of the basophilic bodies of the cytoplasm, loss of glycogen, and accumulation of sudanophilic lipids. These changes in the protected livers are reversible, but they suggest that there is an incipient injury which brings about a transient disturbance in the metabolic activities of the cells.

The mitochondria in unprotected livers become transformed into swollen spheres, whereas those in livers protected by sulfaguanidine retain their normal filamentous form. There is a dissolution of nuclear deoxyribonucleic acid in the prenecrotic centrolobular cells, but the nuclei are unaffected in sulfaguanidine-protected animals. Since damage to the mitochondria precedes that to the nucleus, the mitochondria appear to be the essential target of carbon tetrachloride-induced irreversible cell injury. The mechanism by which sulfaguanidine prevents this attack on the mitochondria is not known.

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Occlusive Disease of the Abdominal Aorta

Structural Alterations

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The now classic report of Leriche defined the clinical manifestations of the syndrome bearing his name.[1] However, morphologic observations on his material were limited. The now established treatment of thrombotic occlusive disease of the abdominal aorta is by resection and replacement with vascular substitutes. This provides an opportunity for detailed study of the changes occurring in such aortas.[2] This study is concerned with the structural alterations observed in the surgically excised segments of 45 abdominal aortas.

Material and Method

All resected specimens included the bifurcation of the aorta, with segments of varying length of the common iliac arteries. The specimens were fixed in 10% formalin, washed in tap water, dipped in 80% ethyl alcohol to restore the color, and then photographed. After gross description of the specimens, portions were taken for microscopic study. Some were cut longitudinally, exposing the lumen of the aorta and common iliac arteries. From other specimens cross sections were taken from the abdominal aorta at the level of the inferior mesenteric artery, from the bifurcation, and from the common iliac arteries. When available, the material removed by thromboendarterectomy from the proximal aorta and distal iliac arteries was also examined. Paraffin or celloidin sections were cut serially from each block and stained, respectively, with hematoxylin and eosin, Masson's trichrome, and Weigert's elastic-tissue stain.

Results

All of the patients (43 white and 2 Negro) were men. Three patients were in the fourth; fourteen, in the fifth; twenty-three, in the sixth, and five, in the seventh decade of life. The youngest patient was 33, and the oldest, 66 years.

Among the 45 patients the occlusion of the aorta was clinically complete in 26 and incomplete in 19. Among those with incomplete occlusion, the right or left common iliac artery was completely occluded in nine, the right being occluded in four and the left, in five instances. In all specimens the occlusive process was due to atherosclerotic change in the walls of the aorta and common iliac arteries. Furthermore, there was some difference in the distribution of atherosclerotic change between specimens from patients with incomplete occlusion and from those with complete occlusion.

In the group with incomplete occlusion the lesions were more extensive and more diffusely distributed in the resected portion of the aorta than in those with complete occlusion, although the roentgenographic appearances suggested involvement primarily of one common iliac artery.

In the specimens with complete occlusion the changes were more marked, more extensive, and apparently older in the common iliac arteries and at the bifurcation than in the more proximal portion of the abdominal aorta. Usually the atherosclerotic and calcific changes in the intima and media narrowed the lumina of the common iliac arteries up to one-third their original caliber. The thrombotic material adherent and contiguous with the changed intima often revealed organization of the coagulum that formed the base for the more recent occluding thrombus. The atheromatous and cal-

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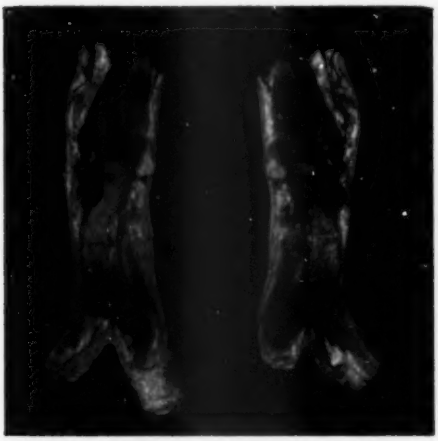


Fig. 1.—Resected distal segment of the abdominal aorta with the common iliac arteries attached, opened longitudinally (Case 1). The lumen is filled with a laminated thrombus. The wall is thickened with atheromatous and calcific plaques.

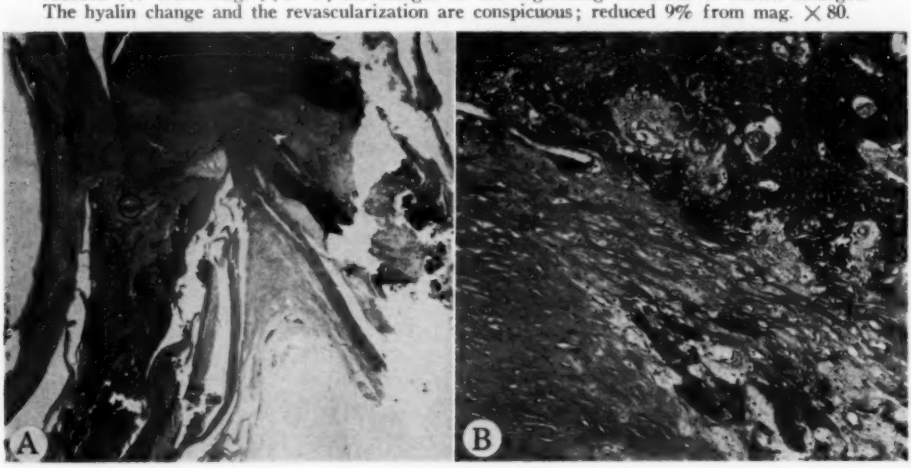
cific change in the wall of the aorta proximal to the bifurcation usually occupied part of the circumference of the vessel wall, and the occluding thrombus seemed more recent. This was evidenced by lack of organization and preservation of the cellular elements and structure of the thrombus. In addition to the atherosclerotic changes in the walls of the iliac arteries and abdominal aorta, there was noted a chronic inflammatory process in the media and adjacent adventitia, evidenced by infiltrations with lymphocytes, plasma cells, and large mononuclear cells.

The following reports are illustrative of the clinical manifestations, operative treatment, and structural changes in the resected portions of the abdominal aorta and common iliac arteries.

CASE 1.—A 52-year-old white man was admitted to the Methodist Hospital on June 2, 1954, with intermittent pain in the lower extremities and sexual impotency. An aortogram revealed complete obstruction of the abdominal aorta and the right iliac artery and almost complete obstruction of the left common iliac artery. The VDRL test of the blood was negative. On June 7 the occluded segment of the aorta was resected and replaced by an aortic homograft. A bilateral sympathectomy was also performed. There was good restoration of the circulation through the homograft, as well as through the iliac arteries. The postoperative course was uneventful, and at the time of discharge there were bounding pulses in the lower extremities.

The specimen consisted of the distal abdominal aorta 6 cm. from the bifurcation. There was 1.5 cm. of the left and 2 cm. of the right common iliac arteries attached. On frontal cut surfaces the entire length of the lumen was filled with a laminated thrombus (Fig. 1). The wall was thickened with atheromatous and calcific plaques.

Fig. 2.—*A*, microscopic appearance of the aorta in Figure 1 at the level of the bifurcation on a longitudinal section. The thrombus immediately above the bifurcation is more recent. The sclerotic and calcific changes are more marked in the common iliac arteries than in the aorta; reduced 9% from mag. × 5. *B*, the margin of the organizing thrombus is shown enlarged. The hyalin change and the revascularization are conspicuous; reduced 9% from mag. × 80.



Microscopically, at the bifurcation of the aorta the lumen was filled with a pink coagulum. The thrombus appeared more recent above the bifurcation than below. The sclerotic, calcific, and osseous changes were conspicuous and more marked in the common iliac arteries than in the aorta (Fig. 2*A*). In the periphery newly formed endothelium-lined spaces organizing the coagulum blended with sclerotic areas of the media (Fig. 2*B*). The inner elastic lamina was scarcely discernible. The broadened or narrowed media was focally and diffusely infiltrated with lymphocytes, plasma cells, and large mononuclear cells, some containing brown granules in the cytoplasm. Similar cells infiltrated also the adjacent adventitia.

Case 2.—A 63-year-old white man was admitted to the Methodist Hospital on Aug. 2, 1954, with pain in the legs of four years' duration. Recently he had been unable to walk more than a block. He stated that he had had an attack of coronary occlusion in 1929, from which he recovered without residual symptoms. He remained well until seven years ago, when he had recurrence of pain. At this time he was thought to have angina and was given belladonna and aminophylline, which he continued to use. There were no pulses felt in the vessels of the lower extremities, including

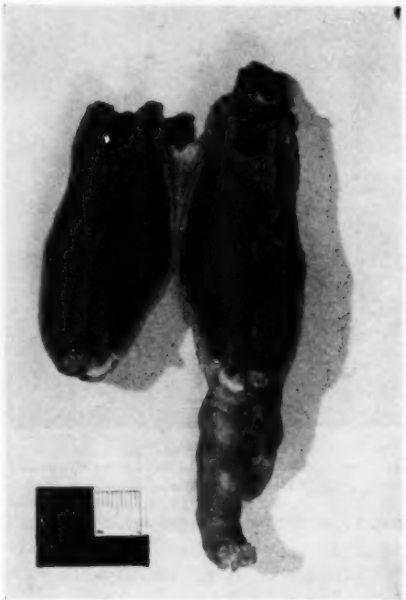
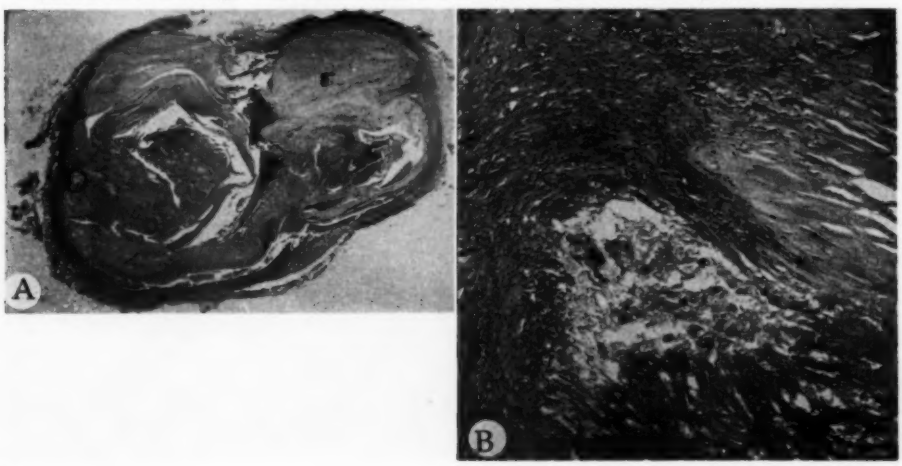


Fig. 3.—Resected distal segment of the abdominal aorta with the common iliac arteries attached, opened longitudinally (Case 2). In the center of the aorta there is a fusiform enlargement. The lumen is filled by a laminated thrombus, with a narrow tunnel piercing the clot eccentrically.

Fig. 4.—*A*, microscopic appearance of the aorta in Figure 3 on cross section at the bifurcation. The asymmetrical narrowing of the lumina of the common iliac arteries by sclerotic broadening of the intima and media involve practically the entire circumference of both vessels; reduced 6% from mag. × 5. *B*, the changes in the iliac arteries are more extensive than is evident in the aorta. The focal chronic inflammatory reaction with infiltration of lymphocytes, plasma cells, and large mononuclear cells is shown; reduced 6% from mag. × 80.



both femoral arteries. A systolic murmur was heard in the midabdominal region. The VDRL test of the blood was negative. An aortagram revealed complete obstruction of the terminal portion of the abdominal aorta just above the bifurcation and extending almost to the renal arteries. On Aug. 11, the occluded abdominal aorta and common iliac arteries were resected and replaced with an aortic homograft. Bilateral sympathectomy was also performed. Convalescence was uneventful, and there was good restoration of the circulation in the lower extremities.

The specimen consisted of a distal abdominal aorta 7 cm. to the bifurcation. The attached portion of the left common iliac artery was 2.5 cm. and of the right, 4 cm. The arteries were firm and beaded, with their walls thickened and the lumina narrowed. In the center of the aorta there was a fusiform enlargement, 3×3 cm. From the proximal lumen protruded a blood clot. On cut surfaces the wall was distorted, in places, discolored yellow; elsewhere it was thinned to 0.1 cm. The lumen was filled with a laminated thrombus, with a narrow tunnel eccentrically piercing the clot (Fig. 3).

Microscopically, at the bifurcation of the aorta the lumen was filled with a pink coagulum composed in its recent portions of ghosts of erythrocytes, platelets, and fibrin. This blended into a more homogeneous coagulum and hyalinized portions containing focal areas of blue granular calcific material, replacing the intima and parts of the media. The media was narrowed or broadened, with the inner elastic lamina scarcely discernible in places (Fig. 4*A*). In the media and intima were focal and diffuse infiltrations of lymphocytes, plasma cells, and large mononuclear cells (Fig. 4*B*). Similar cells infiltrated also some of the adjacent adventitia.

CASE 3.—A 51-year-old white man was admitted to the Methodist Hospital on Oct. 21, 1954, with pain in both legs for the past three years, more marked while walking, burning pain in the hips and penis, and sexual impotency. The symptoms had been progressive, and at the time of admission the patient was incapacitated. The Kolmer test of the blood was negative. An aortagram revealed obstruction of the aorta along its entire length from the renal arteries, including the iliac arteries. On Oct. 26, the abdominal aorta was resected with thromboendarterectomy proximally and distally. The defect was bridged by a lyophilized homograft, and a bilateral sympathectomy was performed. After operation pulsation was restored in the arteries of both lower extremities.

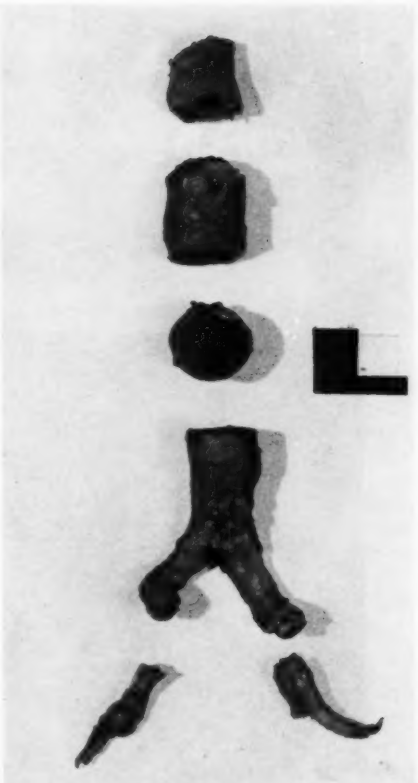


Fig. 5.—Resected distal segment of the abdominal aorta with the common iliac arteries attached and the portions of the intima with thrombi from the distal cut end of the aorta and from each of the common iliac arteries (Case 3). A thrombus fills the lumen.

The specimen consisted of the distal portion of the abdominal aorta 8 cm. to the bifurcation and 3 cm. of common iliac arteries attached. There was a portion of intima 2 cm. long from the distal cut end of the abdominal aorta and similar intimal portions of each of the iliac arteries 3 cm. long. The lumen of the aorta as well as of each common iliac artery was obliterated by a thrombus (Fig. 5). The wall was of uneven thickness, with areas of atheromatous change.

Microscopically (Fig. 6), the lumen of the common iliac arteries at the bifurcation of the aorta was diminished by eccentric thickening of the intima and media and was filled with a clot in places firmly adherent to the intima. Calcific deposits and bony lamellae were distributed around the

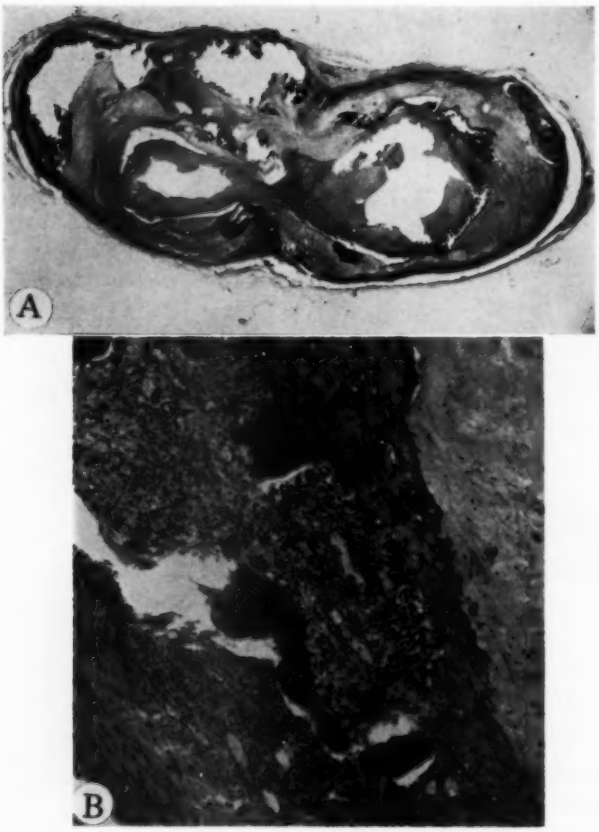


Fig. 6.—*A*, microscopic appearance of the common iliac arteries at the bifurcation of the aorta in Figure 5. There is narrowing of the lumina by eccentric thickening of the intima and media; × 5. *B*, calcific deposits and bony lamellae are distributed around the entire circumference, leaving in places a narrow rim of intact media; × 80.

entire circumference. The media was diffusely infiltrated with lymphocytes, plasma cells, and large mononuclear cells. In few areas the inner elastic lamina could be made out. The media was scarcely recognizable in the areas adjacent to the calcific and osseous deposits. In the adventitia were slight infiltrations of lymphocytes, plasma cells, and large mononuclear cells.

Case 4.—A 43-year-old white man was admitted to the Methodist Hospital on Oct. 26, 1954, with pain in the legs, thighs, hips, and buttocks and sexual impotency. The symptoms had been progressive, and at the time of admission the patient was incapacitated. There were no pulses in the lower extremities. There was pulsation in the epigastrium, but the lower abdominal aorta did not pulsate. There was an audible bruit in the epigastrium. The VDRL test of the blood was negative. An aortagram revealed complete obstruction of the abdominal aorta, beginning at the renal arteries. On Oct. 28, the abdominal aorta was resected and replaced by a lyophilized aortic homograft, and a limited bilateral lumbar sympathectomy was performed. At the time of discharge from the hospital, one week later, there were excellent pulses in both lower extremities.

The specimen consisted of the distal abdominal aorta, 5.5 cm. to the bifurcation, with 3 cm. of the right and 2 cm. of the left common iliac arteries attached. The lumen was filled with a thrombus. The wall was of uneven thickness, with areas of atheromatous change (Fig. 7).

Microscopically, at the bifurcation of the aorta the thrombus in the right common iliac artery appeared older, with calcific deposits extending into the center of the occluding thrombus. The intima was markedly broadened and hyalinized and contained areas of calcific debris. The media was narrowed or broadened. In the periphery of the media there were diffuse infiltrations with lympho-

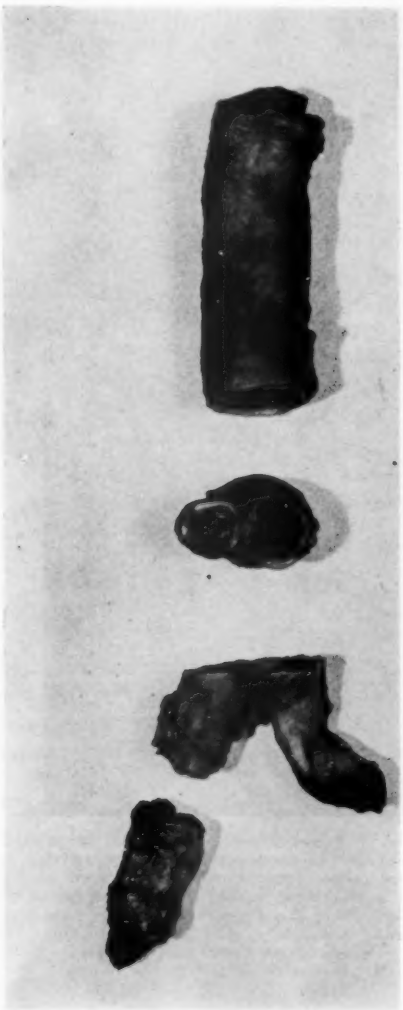


Fig. 7. (Case 4)—Resected segment of the abdominal aorta with portions of both iliac arteries attached. The lumen is filled with a thrombus.

cytes, plasma cells, and large mononuclear cells. The inner elastic lamina could be seen in some fields. The adventitia in focal areas was infiltrated with similar cells (Fig. 8).

CASE 5.—A 52-year-old white man was admitted to the Methodist Hospital on Oct. 26, 1954, with intermittent pain in the calves, thighs, hips, and buttocks and sexual impotency. He had previously had bilateral sympathectomies, with little, if any, relief of symptoms. There were no pulses in the femoral triangles or in the lower extremities. There was an abdominal aortic pulse, and a bruit was heard in the lower abdomen. The VDRL test of the blood was negative. A lumbar aortagram revealed complete occlusion of the distal abdominal aorta and both common iliac arteries. There was partial occlusion of the proximal abdominal aorta from the level of the renal arteries. On Oct. 29, the distal abdominal aorta and common iliac arteries were resected with thromboendarterectomy to the level of the renal arteries. The aorta was replaced with a lyophilized aortic homograft. After the operation satisfactory pulses were felt in the arteries of the lower extremities.

The specimen consisted of the distal abdominal aorta, 4.5 cm. to the bifurcation, with 3 cm. of the right and 1.5 cm. of the left iliac arteries attached. The lumen was filled with a thrombus, with a slit-like space in the center. The thrombus was attached to the vessel wall that was of uneven width, having atheromatous and calcific plaques along the whole length. There was an aneurysmal dilatation of the right common iliac artery, with a thrombus filling the lumen (Fig. 9).

Microscopically, on a longitudinal section at the bifurcation of the aorta the lumen was occluded with erythrocytes or their ghosts in fibrin, adherent to the intima. The intima was broadened and in places replaced by a pink coagulum containing numerous cholesterol clefts. Elsewhere were calcific deposits. The media was narrowed or broadened, and in some fields the inner elastic lamina could scarcely be made out. The media was diffusely infiltrated with lymphocytes, plasma cells, and large mononuclear cells, and in one area there were numerous foam cells; around slit-like cholesterol clefts were giant cells of the foreign-body type. In the adventitia were focal infiltrations of lymphocytes, plasma cells, and large mononuclear cells (Fig. 10).

Comment

Atherosclerotic involvement of the abdominal aorta is usually described as increasing in extent and severity toward the bifurcation. Apparently in patients with occlusive disease the atherosclerotic change commences in the common iliac arteries and diminishes upward past the bifurcation. In patients with incomplete occlusion the

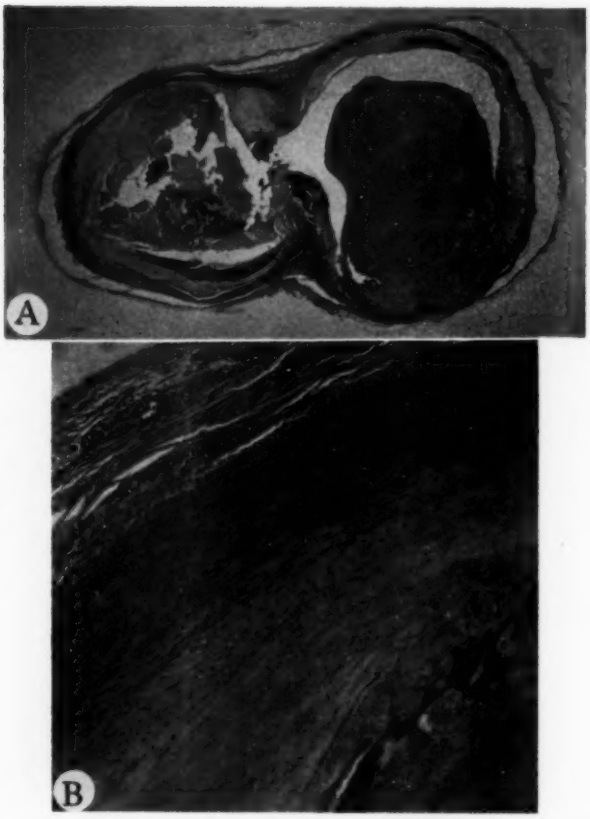
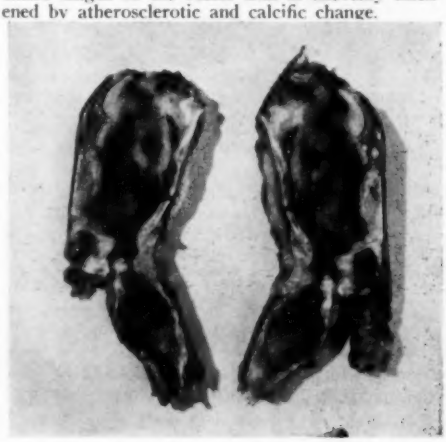


Fig. 8.—*A*, microscopic appearance of the aorta in cross section at the bifurcation in Figure 7. The thrombus in the right common iliac artery appears older, with calcific deposits extending into the center of the occluding thrombus. The clot in the left common iliac appears to be more recent. The vessel walls are unevenly thinned or broadened by atheromatous hyalin change with plaques of calcific deposits; × 5. *B*, in a segment of the right common iliac artery, the remains of the inner elastic lamina are seen. Infiltrations with lymphocytes, plasma cells, and large mononuclear cells are extensive in the media and adventitia; × 80.

change in the abdominal aorta might well be a part of atherosclerotic change in all the arteries. Here, too, as in complete occlusion, the obstructive phenomenon commences in one of the iliac arteries and progresses upward.

Usually the occlusion that produces the Leriche syndrome apparently begins below or at the bifurcation of the aorta at a time when atherosclerotic changes may be as yet insignificant elsewhere. In addition to the atherosclerotic change, a chronic inflammatory reaction, an arteritis, was noted in all of the vessels examined. The exact role of this inflammatory reaction in the changes that lead to the obstructive phenomenon is not clear, though it may have an important part in precipitating the occlusion.

Fig. 9 (Case 5).—Resected distal segment of the abdominal aorta with both iliac arteries attached opened longitudinally. The lumen is filled with a thrombus, leaving a slit-like space in the center. There is a fusiform dilatation of the right common iliac artery, filled with a thrombus. The entire length of the vessel wall is unevenly thickened by atherosclerotic and calcific change.



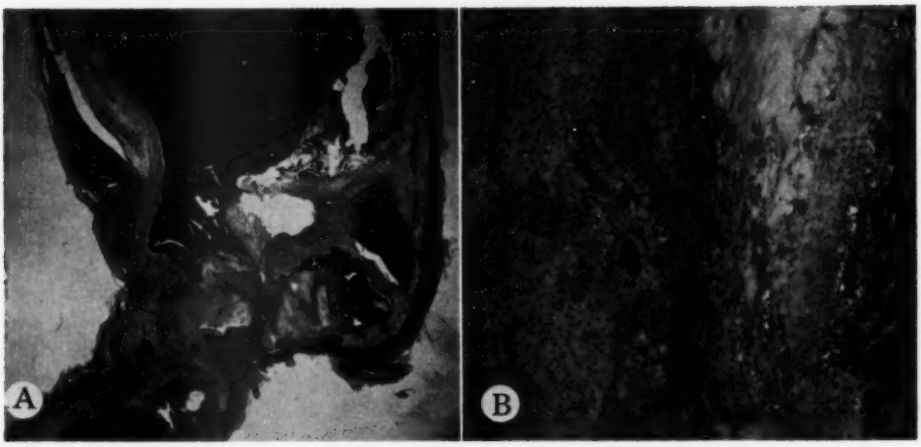


Fig. 10.—*A*, microscopic appearance of the aorta in Figure 9 on longitudinal section from the bifurcation. The atheromatous, sclerotic, and calcific changes are conspicuous. Part of the occluding thrombus appears recent; reduced 9% from mag. × 5. *B*, infiltration of the media with lymphocytes, plasma cells, and large mononuclear cells is extensive; reduced 9% from mag. × 80.

Summary

The structural changes occurring in the abdominal aorta and common iliac arteries in occlusive disease were studied in 45 surgically removed specimens. It was observed that the alterations, atherosclerotic, calcific, and bone formation with a constant focal or diffuse chronic inflammatory reaction, commenced in the common iliac arteries and decreased in severity and extent upward from the bifurcation of the aorta. It is suggested that the inflammatory process observed may have a part in precipitating the occlusion.

Veterans' Administration Hospital, 2002 Holcombe Blvd. (31).

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The Tuberous Sclerosis Complex

A Study of a New Case

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In December, 1956, we had the opportunity to study clinically and at autopsy a case of the tuberous sclerosis complex. The patient was seen at the Hiroshima Citizens Hospital and was subsequently autopsied at the Hiroshima Atomic Bomb Casualty Commission. She had not been exposed to the explosion of the atomic bomb and was accepted as a control case. The extensive reports of Moolten,[1] in 1942, and of Inglis,[2] in 1954, were studied, and the "neural crest" theory of origin postulated by the latter has particularly prompted this case report. Furthermore, the finding of lesions in the lung, adrenal gland, mesentery of the large bowel, and heart, as well as the unusual size of the kidney lesions, make our case a valuable addition to the literature of the tuberous sclerosis complex.

Report of a Case

The patient was a 25-year-old Japanese woman who had been seen as an outpatient by two of us because of a fine papular eruption in a symmetrical distribution over her cheeks which she had had as long as she could remember. A clinical diagnosis of adenoma sebaceum, or "Pringle's disease," was made. In December, 1956, she suddenly developed hematuria, with severe low abdominal colicky pain, and was seen and hospitalized at the Hiroshima Citizens Hospital. Catheterization of both ureters was performed immediately under the diagnosis of renal bleeding. Hematuria was noted from both renal pelves. Retrograde pyelography was performed with pneumoretroperitoneum, and this revealed the appearance of bilateral renal tumors and a suggestion of bilateral polycystic disease of the kidneys was made. Hematuria continued and became severer, and on the sixth day she lapsed into shock and developed epileptic seizures. In spite of emergency treatment, she died on the sixth day of hospitalization as a result of continued severe loss of blood.

A review of the clinical history of the patient revealed that she had had epileptiform attacks several times when she was in elementary school. As a student she had been considered to be below average or backward, and her facial expression was described as being unintelligent.

At autopsy the presence of a symmetrical fine facial eruption was confirmed, and tumors in both kidneys were encountered. The microscopic examination of the renal tumors, of the skin of the face, of the heart, of a small area in the left upper lung lobe, of the mesentery of the large bowel, and of one of the adrenal glands revealed histological features of the tuberous sclerosis complex. The diagnosis may be made whenever two or more of the following components are found: Pringle's adenoma sebaceum, hamartoma of the kidney, tuberous sclerosis of the brain, rhabdomyoma of the heart, and phacoma of the retina. The disease is ordinarily familial, and its genetic behavior is that of a Mendelian dominant. Some cases do occur without any apparent hereditary history.

The cutaneous lesions, or adenoma sebaceum, are seen in about half the cases of the tuberous sclerosis complex. Moolten suggests that this lesion is also essentially a hamartoma and should be called "hamartoma pilosebaceum." The distribution on the face is of the butterfly type, and the lesions are most numerous on either side of the nose and also converge below to the chin. In the present case the section from the skin of the face showed a moderate increase in the number of sebaceous glands and a marked increase in the number of

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hair follicles. Some of the latter were deformed and appeared abortive.

The renal tumors can be found in more than one-half of the persons who have the tuberous sclerosis complex. Usually, however, they are small and not important clinically. They generally do not show signs of very active growth, and a true neoplastic growth of these hamartomas is extremely rare. They generally fulfill the definition of hamartoma because they are nonencapsulated abnormal growths of multiple elements which in a normal form could be expected to occur in the organs involved.

In this case the right kidney weighed 770 gm. and the capsule stripped with slight difficulty. The external surface showed a mass of friable yellow-and-white evidently neoplastic tissue which was irregular in shape. The cut surface showed a large mass of similar tissue which contained one cystic cavity filled with coagulated blood, and this cavity was separated by thin fibrous tissue from the renal pelvis. Only small areas of apparently normal renal parenchyma were noted. In these areas the corticomedullary junction was distinct and the cortex measured 0.5 cm. in thickness. Other areas of the renal parenchyma were separated by large sheets of the abnormal tissue described above. There were thrombi in the vessels in the surviving renal parenchyma and in the abnormal tissue. The greatest dimension of the right kidney was 25 cm., and the largest single nodule had a greatest dimension of 12 cm. The renal pelvis was compressed by the lobulated yellow-and-white tissue, and the mucosa of the renal pelvis showed hemorrhagic areas. The ureter contained an abundant amount of coagulated blood. The left kidney weighed 270 gm., and its capsule was stripped with slight difficulty. Several grayish-white and yellow nodules were seen on the external and on the cut surfaces. The corticomedullary junction was distinct, and the renal pelvis was apparently normal except for a few hemorrhagic areas in the mucosa. The ureter was empty. The cortex measured 0.5 cm. in thickness.

Microscopically, the sections from the two kidneys showed a similar pattern (Figs. 1 and 2). In areas of the parenchyma not

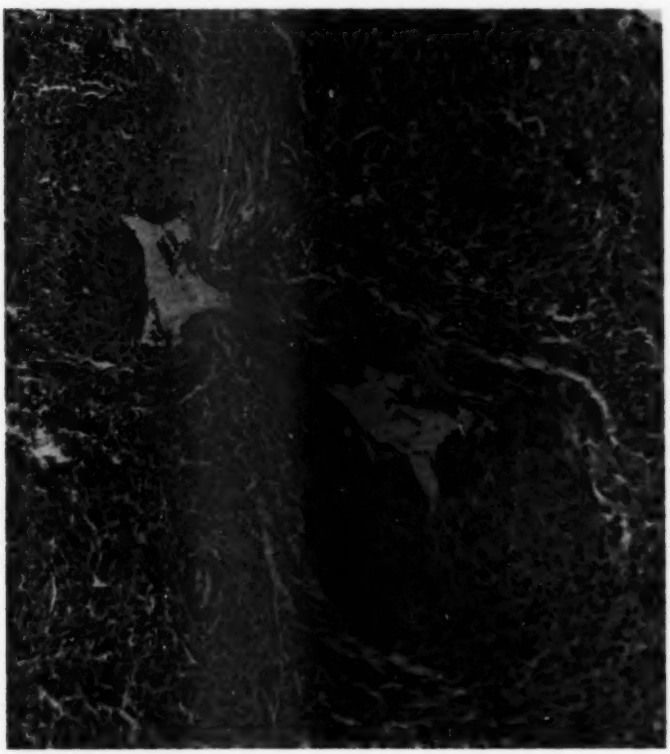

Fig. 1.—Kidney. The atypical arteries isolated in whorled bundles of smooth muscle and fibrocytes were frequently seen throughout the kidney lesions; reduced 13% from mag. × 100.

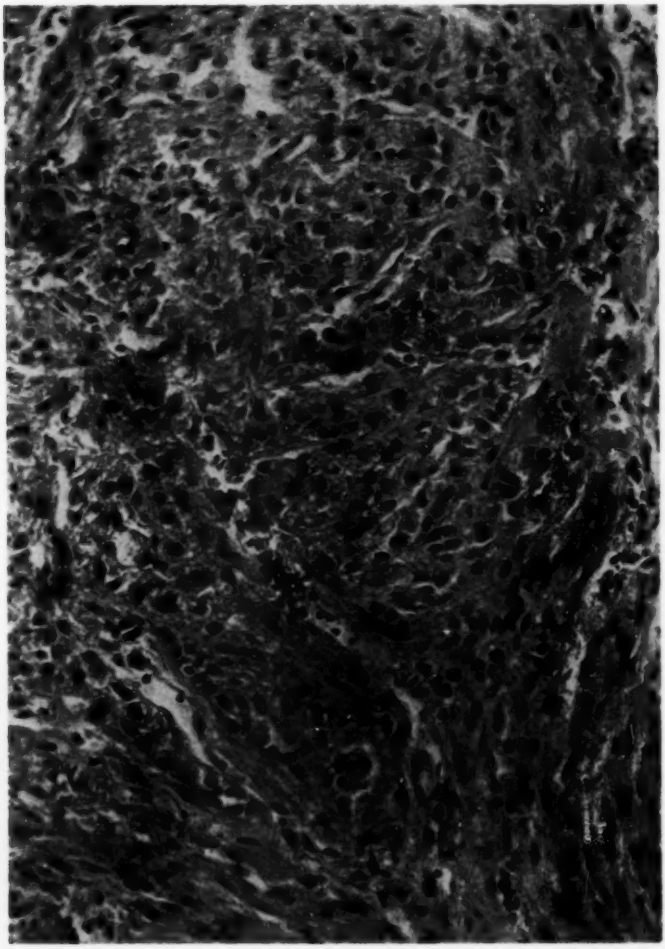

Fig. 2.—The stroma of the kidney lesions resembling smooth muscle and fibrous tissue; reduced 13% from mag. × 200.

directly affected by the tumors described grossly, the renal pattern was essentially normal. Adjacent to and surrounding these areas and not separated from them by either a real or pseudocapsule were the tumor areas. These were made up of whorls and streams of spindle-shaped cells, the nuclei of which usually showed blunt ends and resembled smooth-muscle cells. Some of the nuclei were more fusiform and pointed and resembled fibrocytes. These stromal cells contained a fine sprinkling of Best's-carmine-positive material. Van Gieson stains showed a delicate red network of collagen, with the bulk of the intervening cells not showing any evidence of collagen production. Most of the cells and fibers stained a pale yellow color with Van Gieson stain. Scattered irregularly in this stroma were numerous blood vessels which generally resembled arteries and in which the intima was usually very thick and the media and adventitia were usually incomplete and of varying width. Some of the vessel walls were hyalinized. The lumina of these vessels were usually eccentric. The elastica was scanty or absent. Some islands of fatty tissue (Fig. 3) were isolated in the streams of smooth muscle and fibrous cells. These fatty cells appeared essentially normal. Oc-

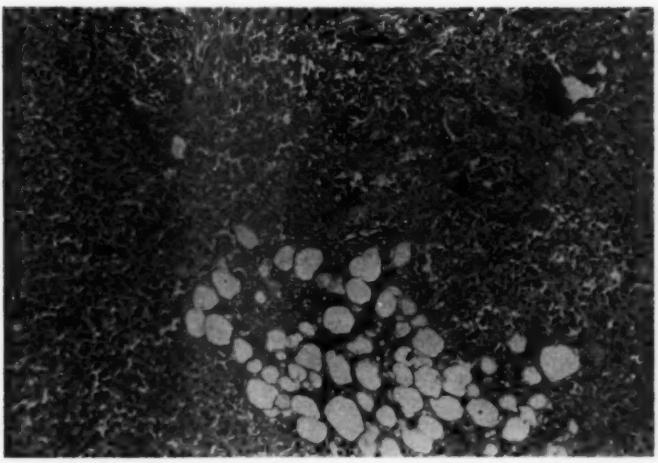

Fig. 3.—Kidney. Another area typical of the renal lesions. An island of apparently normal fat occurring in the fatty and fibrous stroma; reduced 40% from mag. × 100.

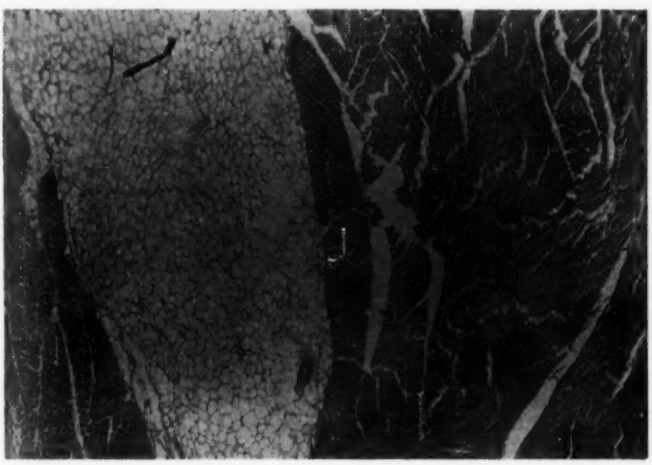

Fig. 4.—An island of apparently normal fat isolated deep in the myocardium of the left ventricle. This was one of several similar lesions in the myocardium; reduced 40% from mag. × 35.

casionally the fibrous cells were moderately atypical, although no mitotic figures were found. In the tumor areas there were large zones of hemorrhage and necrosis accompanied by polymorphonuclear leukocytic infiltration. In the relatively normal renal tissue there were scattered islands of "hamartial germs," usually made up of distorted tubules resting on a smooth muscle and fibrous stroma such as that described above. These islands were of microscopic size.

In many cases gross or histologic evidence of brain disease is not found at autopsy, even though the cerebral manifestations of the disease are its most constant feature. Epilepsy and mental retardation are usually a part of the syndrome. The cerebral lesions may be nodular areas of hardening in the cerebral hemisphere, deformities of entire groups of convolutions, or irregular projections on the inner surfaces of the lateral ventricles. These are usually made up of glial nodules, but neuroblasts are also sometimes present. There appears to be a higher instance of neoplasms of the brain in association with tuberous sclerosis, and Globus [3,4] has suggested that there may be a transition of the hamartial nodules in the brain from a be-

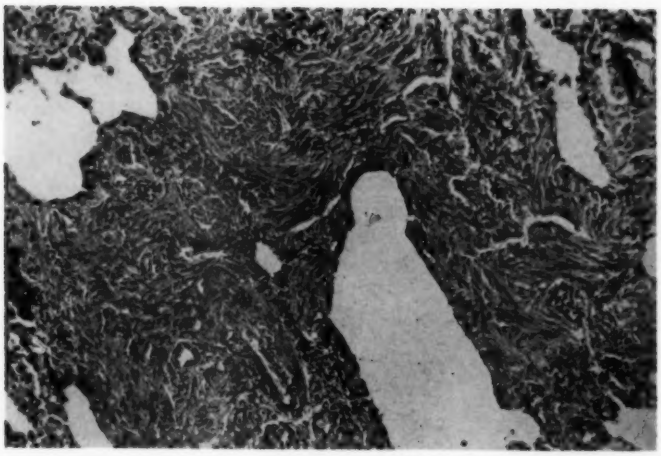

Fig. 5.—Lung. The area is from the left upper lobe and shows the disorderly hyperplasia of smooth-muscle stromal 40% from mag. × 100.

nign to a malignant state. In our case a very thorough examination of the brain, both grossly and microscopically, failed to reveal any lesions.

Rhabdomyomas of the heart have been found in about half the cases in which cerebral changes were also found. In our case only isolated islands of normal-appearing fat (Fig. 4) were found deep in the myocardium of the left ventricle. This fat tissue, like that seen in the kidneys, was completely normal in appearance. We believe that these areas of fatty tissue also constitute "hamartial germs."

In some cases tumor-like lesions of the retina, which have been termed "phacoma," were found. They, as well as the skin lesions, are pathognomonic of the disease. They are found on the disk or separate from it, and their ophthalmoscopic appearance is that of white or gray areas which are round or elliptic. They are hamartomas of glial cells but may also contain a few undifferentiated ganglion cells. Unfortunately, in this case the retina was not examined clinically or at autopsy.

In the left upper lobe of the lung, near a pleural surface but not contiguous with it, a focus of disorderly hyperplasia of smooth muscle was encountered (Fig. 5). This small area resembled the stroma seen

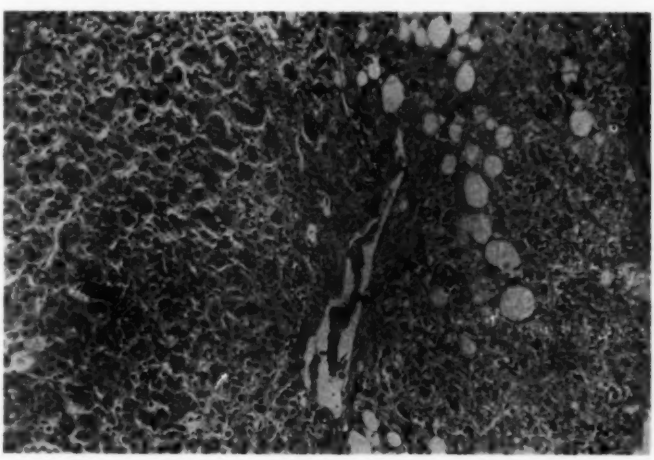

Fig. 6.—Adrenal gland. An island of fatty tissue mixed with fibrous and smooth-muscle stromal elements interrupting the cortex and medulla of the adrenal gland. This lesion closely resembles those seen in the kidneys; reduced 40% from mag. × 100.

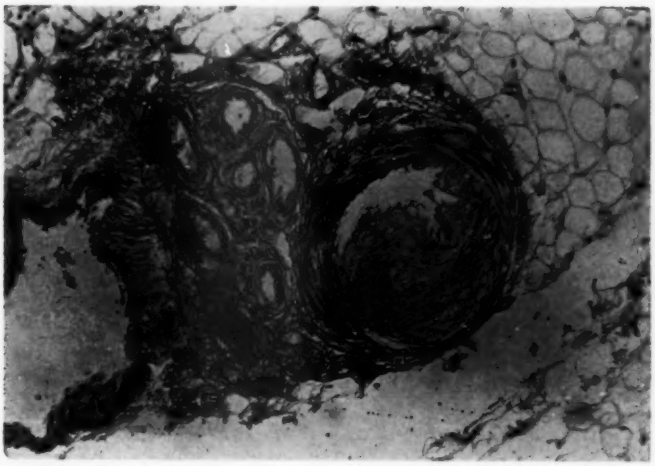

Fig. 7.—The atypical artery in the fatty tissue adjacent to the colon. This artery closely resembles those seen in the kidney lesions; reduced 40% from mag. × 100.

in the renal tumors and was not encapsulated.

In one of the adrenal glands a small area made up of ill-defined stromal cells mixed with normal-appearing fat cells was found. This area interrupted both the cortex and medulla (Fig. 6). The stromal cells resembled smooth-muscle cells in some instances and fibrocytes in others. Some multinucleated forms were seen. These areas in the lung and adrenal gland also apparently represented "hamartial germs."

Careful histologic examination may reveal blood vessel abnormalities in many parts of the body such as those seen and already described in the tumors of the kidneys. Such a finding was made in our case in the fatty tissue of the mesentery of the large bowel (Fig. 7). This lesion consisted of one artery-like vessel which resembled those in the kidney.

Several reported cases [5,6] have had osseous lesions (by x-ray they were islands of increased density alternating with areas of rarefaction) in the skull, hands, and feet. In still other cases a wide variety of associated congenital anomalies has been reported.

In the present case the diagnosis was based on the clinical history of mental retardation and epilepsy, on the gross and histologic appearance of the lesions in the skin of the face, on the hamartomas in both kidneys, on the unusual islands of fat deep in the myocardium of the left ventricle, on the lesion in the adrenal gland, on the focal disorderly hyperplasia of smooth-muscle cells in the left upper lung lobe, and on the abnormal blood vessel in the mesocolon. The islands of fat closely resemble those seen in the tumors of the kidneys. An incidental finding of an active tuberculous lymphadenitis in a pulmonary hilar lymph node was also recorded. An unexplained finding was a marked hyperplasia of the bone marrow, including that of the femoral shaft, predominantly of myeloid cells. There was a marked depletion of fat in the adrenal cortex.

Comment

The nature of the intracerebral and extracerebral tumors in the tuberous sclerosis complex was extensively reviewed by Moolten,[1] in 1942, and again by Inglis,[2] in 1954. Moolten developed the theory that these tumors are hamartial in nature. He applied the principle of embryonic organizers and cell competence to their origin. Under this theory the differentiation of cells in embryonic development depends on two factors, an intrinsic predisposition within the cells to undergo specialization

("cell competence") and an inductive stimulus of chemical nature elaborated by previously specialized cells ("organizers"). He used the primitive lens as an example of the first factor, since it is formed by differentiation of ectodermal cells in response to the inductive stimulus of the outgrowing optic vesicle in the forebrain. The organizer influence depends not only on its own potency but on cell competence as well. The latter decreases as differentiation approaches completion, and the fully differentiated cell may be said to be no longer subject to the influence of organizers. By interfering with the orderly sequence of embryonic organizers, malformations have been produced experimentally. Using this evidence, Moolten postulated that a combination of collections, in mature tissue, of "reserve" cells which are still competent and a faulty organizer action could result in the production of various types of tumors, including the hamartial tumors in the tuberous sclerosis complex. Moolten also submitted that there was much reason to believe that the organizer principle underlies the complication of malignancy in these tumors, especially in the brain.

In his 1954 report, Inglis reviewed the relation of the renal lesions to the cerebral lesions in the tuberous sclerosis complex. In an earlier paper [7] he had hypothesized that there was a common factor underlying all the extracerebral lesions comprised in the tuberous sclerosis complex. He suggested that the cellular tissue in the extracerebral lesions was akin to the neural tissue of neurilemma, or sheath of Schwann, and the extracerebral lesions were considered to belong to a system disease to which the name "neurilemmoblastosis" was given. "Glioneurilemmoblastosis" was suggested as a suitable name when the brain was affected in conjunction with extracerebral lesions. In the paper of 1954, Inglis developed this term, using a case report of the tuberous sclerosis complex in which prominent cerebral and renal lesions were encountered. The cerebral lesions consisted of glial hyperplasia mixed with large ill-defined cells with barely recognizable nuclei. Cytoplasmic strands from these large cells contributed to the glial network. These large ill-defined cells were looked upon by Inglis as early developmental forms which had arisen from the neural tube. The renal lesions in the case varied from 2 cm. in diameter to microscopic size. Adipose tissue constituted a large portion of many of these tumors but in some was inconspicuous. The adipose tissue was sometimes separated by a rather structureless tissue in which there were ill-defined elongated cells with elongated nuclei. These were sometimes mixed with round cells, and there seemed to be an intermediate stage between these two varieties. Multinucleated forms occurred. Inglis felt that these rounded cells in the kidney lesions resembled the ill-defined cells already described in the cerebral lesions. He regarded these cells in both instances as immature cells of neural origin derived from the neural tube. Inglis discussed the presence of apparently normal fat cells in the renal tumors and pointed out that overgrowths of adipose tissue are also found in relation to neurofibroma associated with macrodactyly. He reasoned, "Since lemmocytes, cells of neural crest origin, are the essential element in the formation of neurofibroma (lemmocytoma) with which lipomatous tumors are so closely associated, it is regarded as significant that the lipomatous overgrowths in the renal tumors of the tuberous sclerosis complex are closely associated with tumor cells, of which the advanced forms morphologically resemble the neurilemmal cells of traumatic and spontaneous neurilemmomas, and the immature forms resemble the immature neural cells of the cerebral nodules of tuberous sclerosis. . . ." He also noted that other authors have interpreted these same cellular or nonfatty tumor elements as being fibrous, smooth muscular, or simply "mesenchymal." He admitted that he could not exclude the smooth-muscular possibility on morphologic grounds. He suggested that

if it should prove that the more advanced forms of these cells are smooth muscular rather than neurilemmal, then perhaps neurilemmoblastic cells, involuntary muscle cells, and fat cells all arise independently from the neural crest.

The kidney lesions in our present case had a marked resemblance to those described by Moolten. The blood-vessel abnormalities which he encountered were strikingly similar to those which were present both in the renal lesions and in the one instance in the mesentery of the large bowel in our case. We have not found isolated islands of normal-appearing fat in the myocardium previously described in this condition, nor have we encountered descriptions of the lesions such as we described in the left upper lobe of the lung and in one of the adrenal glands. We are convinced that all of these lesions should be classified as "hamartial germs." It was the 1954 paper of Inglis, however, which stimulated a careful reexamination of our case. Since the lesions in the kidneys which we encountered were so large and so extensive, adequate opportunity should have arisen to confirm the presence of the ill-defined rounded cells which Inglis described in his case and which he postulated were precursors of lemmocytes. Long search has failed to reveal these cells in our case. In addition, there seems to be little doubt that the majority of the stromal cells in these renal tumors are those of involuntary muscle. The microscopic description of our lesions corresponds very well with that of Moolten. We consider that our case supports the hamartial theory of pathogenesis of Moolten, and we further submit that the microscopic appearance of these lesions in our case gives no support to the "neural crest" theory of Inglis.

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The Architecture of the Conduction System in Congenital Heart Disease

I. Common Atrioventricular Orifice

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The position and course of the AV node, bundle, and bundle branches in various congenitally abnormal hearts was systematically investigated by Mönckeberg [1-4] and, to a smaller extent, by Keith.[5]

Mönckeberg found that in patent foramen ovale, subaortic septal defects, and ventricular septal defects situated more distally, the location and direction of the AV node, bundle, and bundle branches were normal. The bundle always passed below the subaortic defect. In complete absence of septa (cor biloculare), the AV node originated from the posterior median atrial tissue in the region of the coronary sinus and the atrioventricular bundle was situated in the posterior wall of the common ventricle. In complete, or almost complete, absence of the ventricular septum (cor triloculare biatriatum), the bundle likewise lay in the median ridge on the posterior wall of the common ventricle. In absence of the interatrial septum (cor triloculare biventriculare), the AV node had the same location as in cor biloculare. The bundle was short, bifurcating early. In patent foramen primum, the AV node likewise originated from the posterior wall of the atrium and the bundle passed along the lower edge of the foramen primum below the atrioventricular valve. In one case of cor biloculare he found an interruption of the bundle between atrium and ventricle. An accessory bundle was present on the left side of the atrioventricular ostium, originating from the muscle of the anterior wall, near the aorta. It passed along the left side of the aorta to join the subendocardial portion of the left ventricle where it divided into two branches. In a case of patent foramen primum; transposition of the ventricles, with both arteries arising from the transposed right ventricle; rudimentary left ventricle, and conus stenosis he also found an anterior accessory connection which joined the main posterior connection which then proceeded downward into the ventricles. Keith [5] likewise found the bundle in ventricular septal defects to lie below the defect.

The conduction system in individual hearts with congenital malformations, with or without known conduction disturbances, have been studied by various authors. Morison,[6] in a case of persistent ostium primum, found the AV node displaced posteriorly and below the coronary sinus. The fibers of the bundle split into an abortive left, which failed to reach the left ventricle, and a large right, which divided further down to supply both the right and left sides. Wilson and Grant,[7] in a case of cor triloculare biatriatum with transposition and pulmonary atresia, associated with 2-to-1 atrioventricular block, found the AV node to be normal. The bundle of His was represented by two strands of tissue coming from the node, entering dense fibrous tissue in the lower

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part of the central fibrous body. This reached the muscular prominence, which was the only remnant of the ventricular septum. Here it became a single narrow band, surrounded and penetrated by fibrous tissue, lying on the surface of this ridge. Four millimeters down, the bundle bifurcated into right and left branches. Yater,[8] in a case of isolated mixed levocardia (transposition of all parts of the heart except the ventricles, with patent ductus arteriosus, foramen ovale, ventricular septal defect in the muscular portion and bicuspid pulmonic valve), associated with complete atrioventricular block, found the SA node in the morphologically right atrium present on the left side and the AV node in the morphologically left atrium present on the right side. The latter was completely separated from the atrioventricular bundle by fibrous tissue. The bundle gave rise to the left bundle branch normally, but the right bundle branch could not be found. Mahaim,[9] in a case of patent foramen ovale and mitral stenosis, associated with left bundle branch (old terminology), found fibrosis of the left bundle branch and inflammatory lesions of the right bundle branch. Yater, Lyon, and McNabb,[10] in a case of large ventricular septal defect in the region of the membranous septum, associated with complete atrioventricular block, found the SA node to be normal. The AV node was atypical, lying on the top of the central fibrous body and deeper within the atrial septum than normal. There was no evidence of an atrioventricular bundle for a large distance; then the bundle commenced in the lower part of the defect and passed to the right on the septal musculature, where it bifurcated into right and left bundle branches. Yater, Barrier, and McNabb,[11] in a case of patent foramen primum with mitral stenosis, associated with complete atrioventricular block, found the AV node displaced posteriorly, not lying adjacent to the central fibrous body. As the central fibrous body got larger the atrioventricular bundle began, and as it reached the defect it presented considerable fibrosis. The bundle branches were normal. Yater, Leaman, and Cornell,[12] in a case of cor triloculare biventriculare, with common entry of the venae cavae and two separate openings for the coronary veins, associated with complete atrioventricular block, found that the AV node originated a short distance below the orifice of the two coronary veins. It gradually became surrounded by fibrous tissue and finally disappeared, except for a few fibers at the atrioventricular junction. There followed a large area where there was no muscular connection, after which the atrioventricular bundle appeared, giving rise to the left bundle branch. The right bundle branch was not found. Wallgren and Winblad,[13] in a case of persistent ostium primum, associated with complete atrioventricular block, found the atrioventricular bundle separated from the AV node for a short distance. Uher,[14] in a case of mixed levocardia (atrioventricular orifices and ventricles transposed), with transposition of the arterial trunks, ventricular septal defect, pulmonary stenosis, and patent ductus, associated with partial heart block, found the SA node to be normal. The AV node originated behind the upper right trigone. The atrioventricular bundle showed an increase in connective tissue, and it bifurcated at the ventricular septal defect into transposed bundle branches. Aschoff [15] pointed out the importance of the conduction system in the diagnosis of the position of chambers in congenital heart disease. Castoldi,[16] in a case of congenital aneurysm of the pars membranacea, associated with bundle-branch block, found the bundle deviated from its course, the right bundle branch interrupted in two regions, and the left in one region, in its anterior part. Lenègre, Deglaude, and Hazim,[17] in a case of fibrocalcareous subaortic stenosis with patent ductus, associated with left bundle-branch block, found the anterior radiation of the left bundle branch destroyed, while the posterior radiation was caught in the fibrotic process of the subaortic stenosis. Turpin, Lenègre, and Chas-

sagne,[18] in a case of a large defect of the ventricular septum, with a common valve for the posterior mitral and medial tricuspid leaflets, and both atrial appendages opening into the right ventricle, with the left ventricle reduced to an aortic vestibule, associated with complete atrioventricular block, found a rudimentary atrioventricular bundle, with complete absence in continuity between atrial and ventricular musculature. Wallgren and Winblad,[13] in an otherwise normally formed heart, associated with complete atrioventricular block, also found the atrioventricular bundle separated from the AV node. Finally, Coakley [19] and Botti and Visioli,[20] in otherwise normally formed hearts, associated with bundle-branch block, found an absence of the right bundle branch.

Details on Hearts Studied Histopathologically

Specimen No.	Method	Sections, No.	Thickness of Section, μ	Sections, Lost, No.
1 (CAV-4)	Serial	2,829	10	11
2 (CAV-3)	Serial	1,829	10	2
3 (CAV-1)	q. 20 *	349	6	0
4 (CAV-2)	q. 20 *	245	6	0

* Every 20th section used.

In order to study the pathology of the conduction system in congenital heart disease and correlate it with electrocardiographic findings, it was deemed necessary to reinvestigate the course of the conduction system in all types of congenital malformations, with utilization of methods and studies of the normal previously evolved.[21-26] This is the first of such studies, and it deals with common atrioventricular orifice. Subsequent publications will deal with tetralogy of Fallot, ventricular septal defect, tricuspid atresia, aortic atresia, left superior vena cava, and other abnormalities. In the present work we are considering common atrioventricular orifice in its pure form, excluding patent foramen primum.

Materials and Methods

Two entire hearts (Specimens 1 and 2) and the AV node, bundle, and bundle branches of two other hearts (Specimens 3 and 4), all the seat of common atrioventricular orifice, were studied histopathologically by serial section. The conduction system of the two latter hearts were cut in the oblique plane originally devised, and every 40th section was retained and strained. The entire hearts (Specimens 1 and 2) were cut in a plane parallel to the posterior wall, and all sections were retained and stained. All sections of Specimens 3 and 4 were stained alternately with hematoxylin-and-eosin and Weigert-and-Van Gieson stains. In Specimens 1 and 2, every fifth section was stained with Weigert and Van Gieson stain, and the others, with hematoxylin-and-eosin stain. The technical details of these hearts are presented in the Table.

Gross and Microscopic Observations

SPECIMEN 1 (CAV-4).—*Gross Examination* (Fig. 1)

The heart was bizarre in shape. The apex was formed by both ventricles. From the base two arteries emerged a larger one, situated anteriorly and to the left, and a smaller, posteriorly and to the right. The interrelationship of the various chambers was normal.

The right atrium was considerably enlarged. It received the superior and inferior venae cavae and coronary sinus in a normal manner. The Eustachian and Thebesian values were fenestrated. The limbus was well formed, but the septum primum was fenestrated, producing a patent foramen ovale.

In the distal part of the atrial septum and the proximal part of the ventricular septum there was a large opening. This was seen to be a combination of a patent foramen primum and an interventricular foramen. It was thus apparent that there was a common atrioventricular orifice. The right component of this orifice was larger than the left. It was guarded by an inferior and an anterior leaflet of normal contour and normal connections with the anterolateral and inferior papillary muscles. The medial leaflet was divided into two parts. The upper part was continuous with the anterior part of the anterior mitral leaflet, while the inferior part was continuous with the inferior part of the anterior mitral leaflet. Both parts of the medial leaflet were connected by chordae to the septum. There was

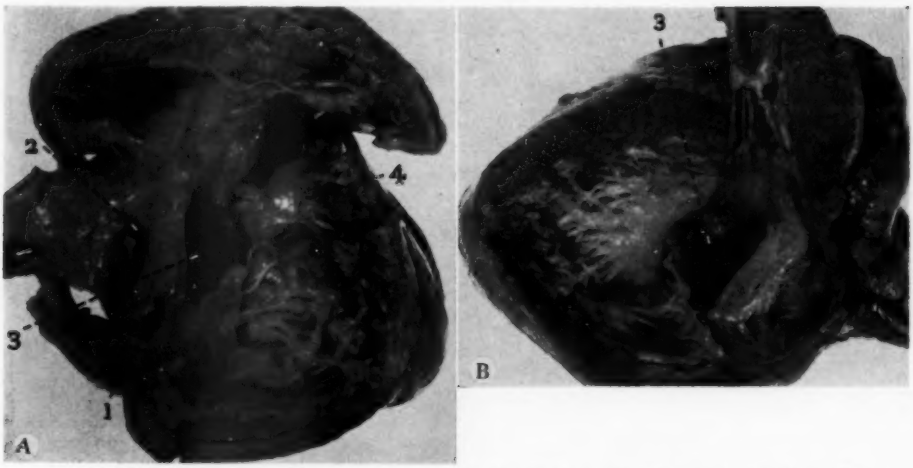


Fig. 1 (Specimen 1).—*A*, right atrial and ventricular view. *B*, left ventricular view. The numbers indicate the parts as follows: 1, entry of coronary sinus; 2, patent foramen ovale; 3, common atrioventricular orifice with patent foramen primum and foramen interventriculare; 4, septal band of crista supraventricularis.

a wide hiatus devoid of valve between the two parts.

The right ventricle was larger than the left, and its wall was as thick as the left. The crista supraventricularis was normal in contour. This chamber gave off the pulmonary artery normally. Its orifice was greater than that of the aorta. It gave off the two secondary pulmonary arteries normally. The ductus arteriosus was widely patent.

The left atrium was relatively small in comparison to the right. It received the four pulmonary veins normally. The left component of the common atrioventricular orifice was much smaller than the right. It was guarded by a cleft anterior and inferior leaflet. The upper and lower poles of the two parts of the anterior leaflet and the inferior leaflet were connected to normal papillary muscles. The medial aspect of the lower part of the anterior leaflet was connected by chordae to the margins of the defect.

The left ventricle was relatively small, and its wall was equal to that of the right ventricle. From this chamber emerged the aorta. The orifice of the aorta was smaller than that of the pulmonary artery. Its valve was normal. The coronary and branchiocephalic vessels were not studied.

Pathologic Diagnosis

The pathologic diagnosis was as follows: (1) common atrioventricular orifice; (2) patent foramen primum and interventricular foramen; (3) patent foramen ovale; (4) right atrial and ventricular hypertrophy; (5) hypoplasia of the left atrium; (6) dilatation of the pulmonary artery; (7) fenestration of the Eustachian and Thebesian valves.

Histologic Examination of the Conduction System

The SA node was found to be normal. The AV node originated in the posterodistal wall of the right atrial musculature, adjacent to the right atrioventricular sulcus and the interventricular groove (Fig. 2*A*). It lay distal to the coronary sinus and adjacent to the base of the muscular right atrial-left ventricular septum. The node penetrated the posterior portion of the central fibrous body, and as it did so it gave off numerous paraspecific communications to the ventricular septum. As it ascended in this body it came to lie adjacent to the annulus of the mitral portion of the common valve (Fig. 2*B*). Here again it gave off numerous paraspecific fibers. It reached the defect in the region of the foramen primum atrioventriculare, where it lay beneath a remnant of the pars membranacea (Fig. 3*A*). It now swung

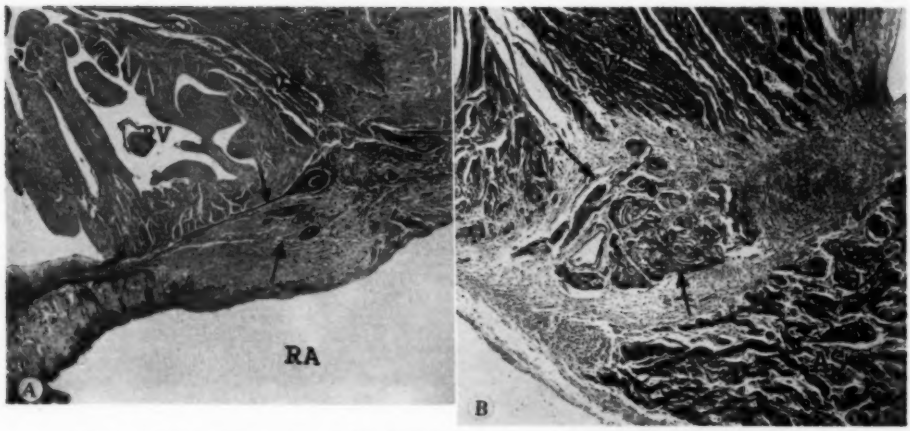
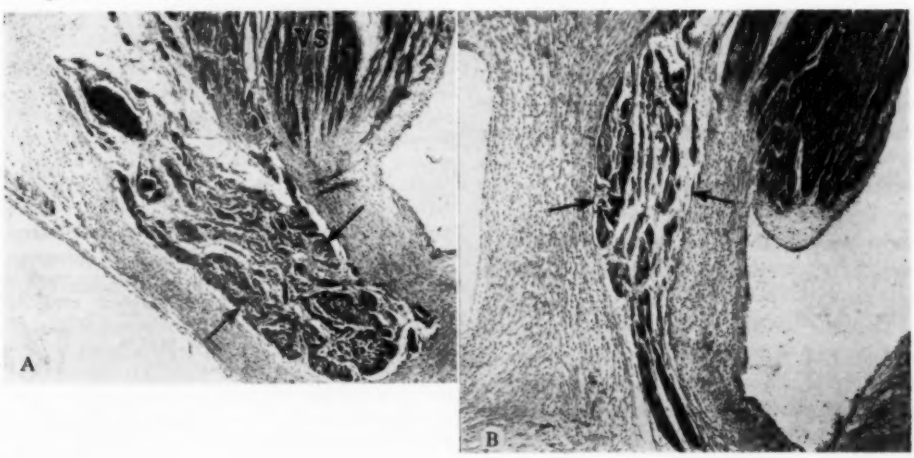


Fig. 2.—Conduction system of Specimen 1. (Note method of sectioning in text.) *A*, atrioventricular node; Weigert and Van Gieson stain; reduced 40% from mag. × 15. *B*, penetrating portion of atrioventricular bundle, lying adjacent to attachment of the annulus of the mitral portion of common valve, hematoxylin and eosin; reduced 40% from mag. × 60. RA indicates right atrium; RV, right ventricle; VS, ventricular septum; arrows point to the node and bundle.

horizontally, skirting the orifice till it arrived at the attachment of the annulus of the tricuspid portion of the common valve (Fig. 3*A*) and hence in the region of the common atrioventricular orifice. Here it gave off more paraspecific fibers. It now continued its trek distally in the remnant of the pars membranacea (Fig. 3*B*), skirting the interventricular foramen until it reached the summit of the posterior ventricular septum. At this point the first fibers of the posterior radiation were given off. Also further paraspecific fibers were given off at this point. Close to the distal wall of the interventricular foramen, it divided into the right and the remainder of the left branch (Fig. 4), that is, the remainder of the posterior radiation and the anterior radiation. The right was a compact bundle which ascended the right side of the septum in a somewhat oblique manner. At one point it sent a strong branch to join the myocardium. Near the muscle of Lancisi it divided

Fig. 3.—Conduction system of Specimen 1. *A*, penetrating portion of atrioventricular bundle lying beneath the foramen primum atrioventriculare; hematoxylin and eosin; reduced 40% from mag. × 60. *B*, atrioventricular bundle lying beneath the interventricular foramen; hematoxylin and eosin, reduced 40% from mag. × 60. VS indicates ventricular septum; arrows point to the bundle.



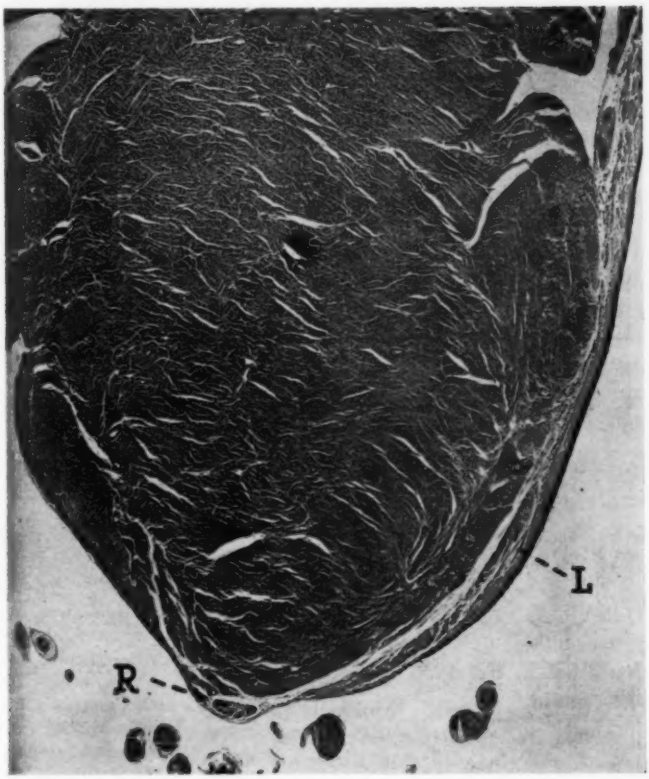


Fig. 4. — Conduction system of Specimen 1. Section shows bifurcation into right and left bundle branches. R indicates right bundle branch; L, left bundle branch. Weigert and Van Gieson stain; × 15.

into numerous fasciculi, which then came together again and joined the musculature of the parietal wall. There was a slight infiltration of mononuclear cells in the termination of the anterior radiation of the left bundle branch.

SPECIMEN 2 (CAV-3).—*Gross Examination* (Fig. 5)

The heart was minute. The apex was formed by the left ventricle. From the base, the two arteries emerged in their normal position. The interrelationship of the various chambers was normal.

The right atrium was larger than the left. The superior and inferior venae cavae and

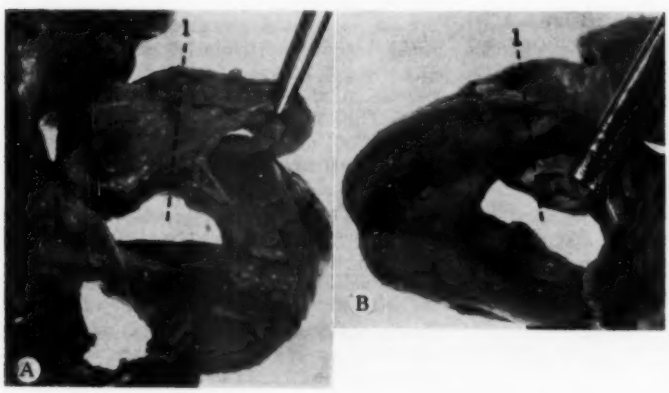


Fig. 5 (Specimen 2).—*A*, right atrial and ventricular view. *B*, left ventricular view. The number 1 indicates combined defect.

coronary sinus entered this chamber normally. There was no Eustachian valve. The Thebesian valve was represented by a band.

A giant opening was present in the atrial and the proximal portion of the ventricular septum. The rim proximally was made up by the limbus and distally, by the basal portion of the muscular ventricular septum. The opening thus consisted of a foramen ovale, a foramen primum, and an interventricular foramen. There was thus one common atrioventricular orifice. The right component of this orifice was guarded by an anterolateral leaflet and an inferior leaflet, which were normal in contour and had normal connections with the anterolateral and the inferior papillary muscles. The medial leaflet was in two parts. The upper portion was continuous with the anterior part of the cleft anterior mitral leaflet, while the lower portion was continuous with the inferior portion of the cleft anterior mitral leaflet. Both portions were connected in the usual way by chordae to the septum.

The right ventricle was somewhat enlarged, and the wall was somewhat thickened. The crista supraventricularis was normal in contour. This chamber gave rise to the pulmonary artery. The circumference of this artery was slightly greater than that of the aorta. It gave off the two secondary pulmonary arteries normally. The ductus arteriosus was open.

The left atrium received the four pulmonary veins normally. The chamber was small. The mitral portion of the common valve consisted of a cleft anterior and a posterior leaflet. The upper end of the anterior and the lower end of the inferior portion of the anterior leaflet and the posterior leaflet were connected to two normally formed but relatively small papillary muscles. The other end of the lower segment of the anterior leaflet of the mitral valve was connected to the margin of the septal defect. Here there was also a hiatus devoid of valve tissue, between the two components of the anterior mitral leaflet.

The left ventricular chamber was larger than the right, and the wall was thicker than that of the right. From this chamber emerged the aorta. The orifice was slightly smaller than that of the pulmonary artery. It was guarded by a normal valve. The coronary arteries were given off normally. The brachiocephalic arteries were normal. The coronary distribution appeared to be normal. The coronary veins were not dissected.

Pathologic Diagnosis

The pathologic diagnosis was as follows: (1) almost complete absence of the atrial septum, with common atrioventricular orifice (cor triloculare biventriculare); (2) absence of the Eustachian valve.

Histologic Examination of the Conduction System

The SA node was found to be normal. The AV node arose from the musculature of the posterior wall of the right atrium adjacent to the atrioventricular sulcus and from the musculature of the most distal portion of the remnant of the atrial septum (Fig. 6). The latter was really a portion of the atrioventricular septum, since it lay between the attachments of the mitral and tricuspid portions of the common valve. The node lay distal to and at the upper margin of the mouth of but not directly adjacent to the coronary sinus. It pierced the posterior component of the central fibrous body to become the penetrating portion of the atrioventricular bundle. It ascended through the posterior component of the central fibrous body, where it gave off paraspecific fibers, to reach the rim of the defect at the level of the atrioventricular orfice. Here it lay in a remnant of the pars membranacea. It now proceeded distally at the base of the common atrioventricular orifice beneath the remnant of pars membranacea, where it took on the morphology of the branching portion of the atrioventricular bundle. As it reached the level of the interventricular foramen it gave off the first fasciculi of the posterior radiation of the left bundle branch. Here it also lay in a remnant of the pars membranacea. At the junction of the bundle and the left bundle branch more paraspecific fibers were given off. In its further journey distalward it lay directly on the summit of the posterior ventricular septum. At this point, part of the right side of the main bundle separated as a fasciculus, running independently through the adjacent portion of the ventricular septum. It then returned to the main

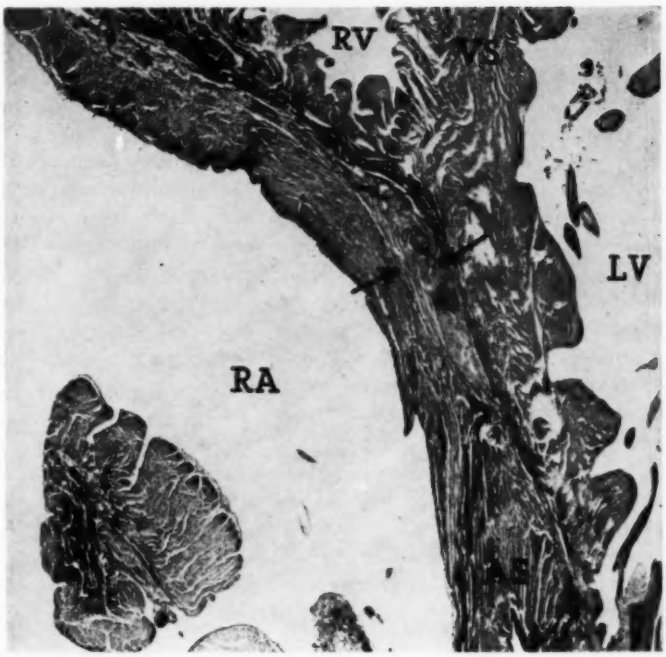


Fig. 6.—Section of AV node of Specimen 2. (Note method of sectioning in text.) RA indicates right atrium; RV, right ventricle; LV, left ventricle; VS, ventricular septum; AS, atrial septum; arrows point to node. Weigert and Van Gieson stain; reduced 15% from mag. × 10.

bundle. At the beginning of the distal wall of the defect, the bundle flattened out as it ascended on this wall, dividing into groups of fasciculi. Several of the fasciculi were given off separately at different levels to form the right bundle branch. One of these groups of fasciculi communicated with the myocardium. The remainder of the left posterior and the anterior radiations were then given off in a continuous streaming of fine fasciculi. The various fasciculi of the right came together again to form a single bundle of divided fasciculi at the region of the muscle of Lancisi. Here communications were given off to the septum. The right bundle branch then proceeded to the moderator band. A band of fibers of the left anterior radiation passed upward almost to the base of the aorta and ended blindly.

SPECIMEN 3 (CAV-1).—*Gross Examination*

The heart was increased in size and abnormal in shape. There was an increase in the broad diameter. The apex was formed by the right ventricle. From the base two arteries emerged, a larger situated anteriorly and to the left and a smaller situated posteriorly and to the right. The interrelationship of the various chambers of the heart was normal.

The right atrium was enlarged, and the wall was thickened. The superior and inferior venae cavae and coronary sinus entered this chamber normally. The coronary sinus was small. The Eustachian valve was small, and the Thebesian valve was absent. The limbus was well formed. The foramen ovale was patent.

Between both ventricles and both atria in the region of the junction of the atrial and ventricular septa there was a large defect. This opening was bounded proximally by the lower margin of the atrial septum, anteriorly by the anterior part of the ventricular septum, distally by the upper margin of the posterior part of the ventricular septum, and posteriorly by the basal portion of the posterior part of the ventricular septum. It was thus apparent that this opening involved the lower part of the atrial septum and the upper part of the ventricular septum.

The tricuspid orifice was seen to be incomplete, being a part of a large orifice which included it and the mitral orifice. The tri-

cuspid component of this orifice was larger than the mitral. The tricuspid component was guarded by an anterolateral leaflet which had the usual morphology and connections of the normal anterolateral tricuspid leaflet. A somewhat enlarged inferior leaflet had the usual morphology and connections of the normal inferior leaflet. The medial leaflet was seen to be divided. The inferior portion was continuous through the defect with the inferior part of the divided anterior mitral leaflet. The superior portion was continuous through the defect with the superior portion of the anterior mitral leaflet. Both portions were connected by the usual chordae to the septum.

The right ventricle was enlarged, and its wall, thickened, equalling that of the left ventricle. The architecture of the sinus and conus was normal. The crista supraventricularis was normal in configuration but hypertrophied. From this chamber emerged the pulmonary artery. The orifice was greater than that of the aorta. The valve consisted of three cusps. The right and left secondary pulmonary arteries were given off normally. The ductus arteriosus was closed.

The left atrium was relatively small as compared to the right. It received the four pulmonary veins normally. The mitral component of the common atrioventricular valve was seen to consist of a cleft anterior leaflet and a small posterior leaflet. The anterior portion of the anterior leaflet was connected to the anterior papillary muscle and by chordae to the common defect. The posterior portion of the anterior leaflet was connected to the posterior papillary muscle and by chordae to the common defect. The posterior leaflet was connected to both the anterior and the posterior papillary muscle, both of which were rather small, especially the inferior.

The left ventricle was small in comparison to the right, and the wall was about the same thickness as the right. From this chamber emerged the aorta. It was guarded by three cusps of normal contour. The wall of the base of the aorta was relatively thin as compared to that at the base of the pulmonary artery. It gave off the brachiocephalic vessels normally. The transverse portion, distal to the innominate, was distinctly narrower than either the ascending or the descending portion. The coronary arteries were given off normally, and their distribution was normal.

Pathologic Diagnosis

The pathologic diagnosis was as follows: (1) common atrioventricular orifice; (2) patent foramen ovale; (3) hypertrophy of the right atrium and ventricle; (4) dilatation of the pulmonary artery; (5) hypoplasia of the left atrium; (6) hypoplasia of the aorta with coarctation.

Histologic Examination of the Conduction System

The AV node arose in the distal portion of the inferior right atrial wall and in the most posterior portion of the atrial septum, at its junction with the two posterior walls of the atria (Fig. 7). It lay distal to the mouth of the coronary sinus, proximal to the attachment of the annulus of the mitral portion of the common valve to the posterior part of the central fibrous body, and at the level of the patent foramen primum. The distal fibers of the AV node streamed superiorly, penetrating the posterior atrial septal extension of the central fibrous body (Fig. 7). Here it lay adjacent to the annular attachment of the mitral portion of the common valve in the floor of the foramen primum, where it assumed the morphology of the atrioventricular bundle (Fig. 8). It now assumed a horizontal course, following the lower boundary of the foramen primum until it reached the level of the attachment of the annulus of the tricuspid portion of the common valve (Fig. 9*A*). It thus now lay at the level of the common atrioventricular orifice. In the proximal part of the common atrioventricular orifice, the bundle lay directly adjacent to the orifice. In the distal part, it lay beneath a remnant of the pars membranacea, where it assumed the morphology of the branching bundle, with fine fasciculi of the left bundle branch beginning to course posteriorly (Fig. 9*A*). Paraspecific fibers were also given off at the junction of the bundle with the left fasciculi. As it reached the level of the interventricular foramen, the bundle shifted to the left, beneath the remnant of the pars membranacea. It now became superficial, giving off many more fibers of the posterior radiation of the left (Fig. 9*B*). It thus reached the distal wall of the inter-

Fig. 7.—Section of AV node of Specimen 3. (Note method of sectioning in text.) The numbers indicate the parts as follows: 1, ramus septi fibrosi; 2, posterior portion of the central fibrous body; 3, right atrial musculature; arrows point to node. Weigert and Van Gieson stain; reduced 10% from mag. × 30.

ventricular foramen, where it divided into the right and the remainder of the left branch (Fig. 10). Both branches swung upward on the distal wall of the interventricular foramen. This portion of the left branch consisted of the termination of the posterior radiation and all of the anterior radiation. The right bundle branch journeyed along the border of the crista, dividing into several fasciculi (Fig. 11), each journeying independently toward the moderator band. Here they came together again.

SPECIMEN 4 (CAV-2).—*Gross Examination*

The heart was enlarged and globular in shape. The oblique diameter was increased. The apex was formed by the left ventricle. From the base two arteries of about equal size emerged, one situated anteriorly and to the left, while the other was posterior and to the right.

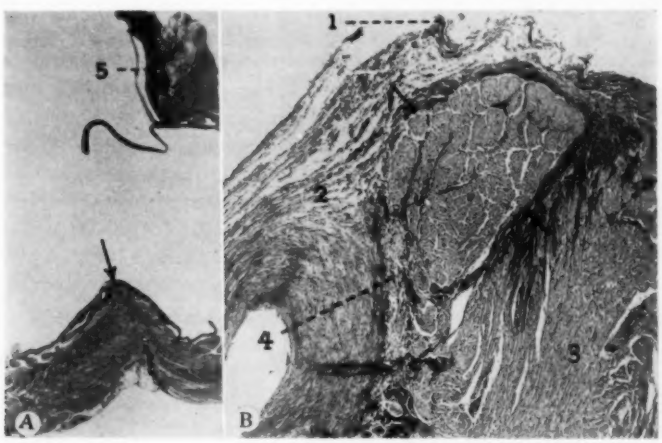


Fig. 8.—Section of penetrating portion of atrioventricular bundle of Specimen 3. *A*, Weigert and Van Gieson stain; reduced 35% from mag. × 4.5. *B*, Weigert and Van Gieson stain; reduced 35% from mag. × 60. The numbers indicate the parts as follows: 1, posterior boundary of patent foramen primum; 2, annulus of mitral portion of common valve; 3, ventricular septum; 4, paraspecific fibers; 5, base of aorta.

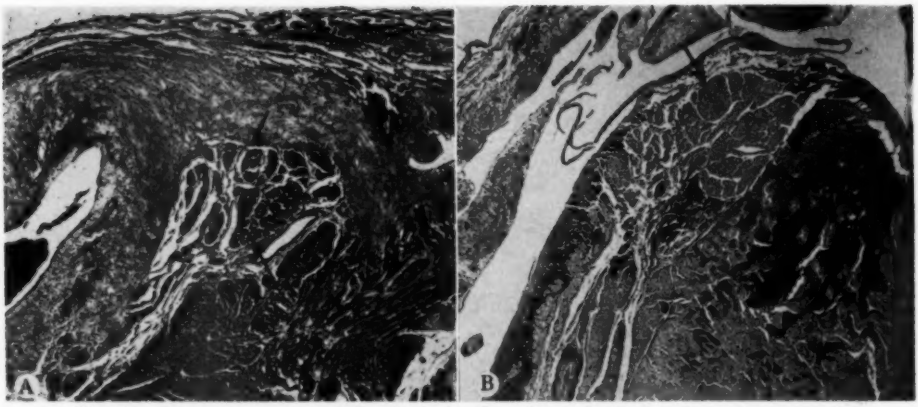


Fig. 9.—Conduction system of Specimen 3. *A*, atrioventricular bundle at the level of the common atrioventricular orifice; hematoxylin and eosin; reduced 40% from mag. ×40. *B*, atrioventricular bundle at level of interventricular foramen; Weigert and Van Gieson stain; reduced 40% from mag. ×60. Arrows point to bundle.

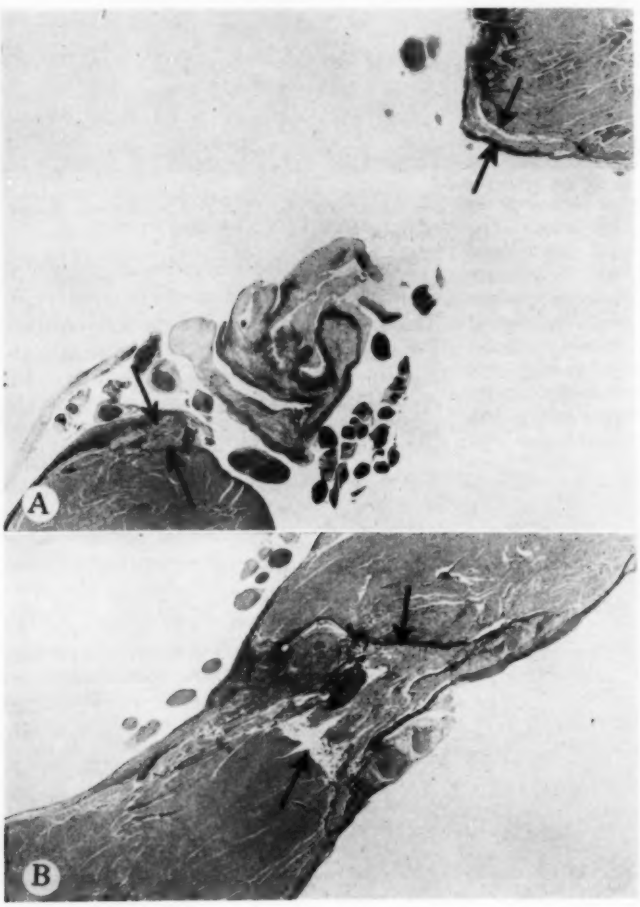


Fig. 10 (Specimen 3).—Section of bifurcation of atrioventricular bundle at the distal wall of combined defect. *A*, at the level of distal portion of defect. *B*, at distal wall of combined defect. Weigert and Van Gieson stain; reduced 10% from mag. ×7.5.

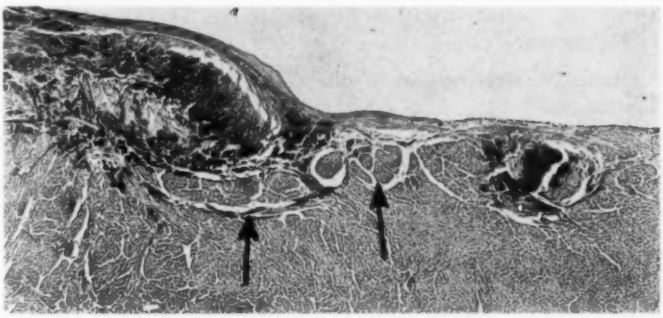


Fig. 11.—Section of the right bundle branch in Specimen 3, in the first part of its course. Note the group of separate fasciculi. Arrows point to the right bundle branch. Weigert and Van Gieson stain; reduced 10% from mag. × 30.

There was one large atrial chamber subdivided slightly by a ridge into a large right and a small left atrium. The superior and inferior venae cavae entered the right atrium normally. Two separate openings of what apparently corresponded to the coronary sinus were evident. No Eustachian or Thebesian valves were present.

The arc-like ridge which divided the atrium into two was seen to be a very wide limbus. A minute band hanging from the upper portion was the only remnant of the septum primum. The limbus became confluent with the upper margin of the muscular ventricular septum to produce a huge opening. The opening was thus between the two atria and the ventricles and was thus a combination of patent foramen ovale, patent foramen primum, and patent interventricular foramen.

It was thus apparent that there was one large common atrioventricular orifice. The right component was guarded by a normal anterolateral leaflet, a normal inferior leaflet, and an abnormal medial leaflet. The latter was split into two, the anterior portion being continuous with the anterior part of the anterior mitral leaflet and the posterior portion being continuous with the posterior part of the anterior mitral leaflet. There was a large gap devoid of valve structure between the two portions.

The right ventricle was large, and the wall was thick. However, it did not equal the left ventricle in thickness. The crista supraventricularis was of normal architecture but hypertrophied. From this chamber emerged the pulmonary artery. The orifice was somewhat smaller than that of the aorta, and the valve was normal. The pulmonary artery gave off the right and left branches normally. The ductus arteriosus was slightly patent.

The drainage of the four pulmonary veins could not be made out from the specimen.

The left component of the common atrioventricular orifice was smaller than the right. It was guarded by a cleft aortic leaflet of the mitral and an inferior leaflet. The anterior portion of the aortic leaflet was continuous with the anterior part of the medial leaflet of the tricuspid, while the posterior portion of the aortic leaflet was continuous with the posterior part of the medial leaflet of the tricuspid. The anterior and posterior papillary muscles were small but normal.

The capacity of the left ventricle was equal to that of the right, but the wall was thicker. From this chamber emerged the aorta. The circumference of the aorta was somewhat greater than that of the pulmonary artery. The valve consisted of three normal cusps. It gave off the brachiocephalic vessels normally. The coronary ostia and distribution were normal.

Pathologic Diagnosis

The pathologic diagnosis was as follows: (1) almost complete absence of the atrial septum, with common atrioventricular orifice (cor triloculare biventriculare); (2) hypertrophy of the right atrium and ven-

tricle; (2) patent ductus arteriosus; (4) double coronary sinus.

Histologic Examination of the Conduction System

The posterior part of the atrial septum was seen to be to the left of the interventricular sulcus and was very small. The AV node originated from the atrial musculature of the distal part of the posterior wall of the right atrium, at the level of the attachment of the annulus of the mitral portion of the common valve to the posterior component of the central fibrous body. It thus originated distal to, but not directly related to, the two coronary sinuses. It penetrated the posterior component of the central fibrous body, thereby becoming the atrioventricular bundle. It now journeyed distally, hugging the inferior border of the atrioventricular defect. At the level of the common atrioventricular orifice, it lay in the posterior component of the central fibrous body, beneath the common atrioventricular valve. Numerous paraspecific fibers were given off in this area. In the most distal part of the common atrioventricular orifice, fibers of the left posterior radiation began to be given off, or these may be considered paraspecific fibers, since they ended close to the summit of the septum. The bundle then reached the level of the interventricular foramen, where it lay beneath the remnant of the pars membranacea on the left side of the posterior septum. In this position it gave off a few more strands of the left posterior radiation. It journeyed in this position to the distal aspect of the interventricular foramen, where it swung upward on its distal wall. At this level it divided into the main portion of the left and the right branches. The left immediately divided into the remainder of the posterior and the anterior radiations which passed to the papillary muscles and trabeculae carneae. The right journeyed over the crista to the region of the muscle of Lancisi. It proceeded distally but could not be completely followed because of the early origin of the moderator band, which was separated from the specimen.

Comment

The course of the conduction system in common atrioventricular orifice is related to the basic anatomy and embryogenesis of this anomaly. The latter have been repeatedly reviewed.[27-33]

In this anomaly there is one common undivided orifice between the atria and the ventricles. This is guarded by a valve consisting usually of four or five leaflets. There may be an anterior and posterior common to both left and right sides of the orifice, with a single lateral on each side of the orifice. Or, in addition to the above, there may be an additional leaflet (sometimes two) on the right side of the orifice. The common anterior leaflet usually represents the upper part of the cleft medial leaflet of the tricuspid fused with the upper part of the cleft anterior leaflet of the mitral. Less commonly, the latter is fused with the anterior leaflet of the tricuspid to form this common anterior leaflet. The common posterior leaflet usually represents the inferior part of the cleft medial leaflet of the tricuspid fused with the inferior part of the cleft anterior leaflet of the mitral. Less commonly, the entire medial leaflet is fused with the inferior part of the cleft mitral valve to form the common posterior leaflet. The two lateral leaflets represent the inferior leaflets of the mitral and tricuspid valves. The anterior leaflet of the tricuspid may be a separate leaflet, or, as seen above, it may be a part of the large common anterior leaflet.

The valve structures representing the anterior and inferior leaflets of the tricuspid have the normal attachments to papillary muscles. That representing the cleft or uncleft medial leaflet of the tricuspid likewise is attached by chordae to the septum as normally. In the left ventricle, however, it is common to have either a small posterior papillary muscle, a ridge-like structure reminiscent of a papillary muscle, or an absence of the posterior papillary muscle. The anterior papillary muscle is always well formed. Thus the anterior portion of the cleft aortic leaflet is attached to the anterior papillary muscle and sometimes by chordae directly to the septum. The posterior portion of the aortic leaflet is attached to the inferior papillary muscle when present and by chordae to the septum. The inferior leaflet is either attached to both papillary muscles or, in the event of absence of the inferior papillary muscle, to only the anterior and by chordae to the septum.

This anomaly is always accompanied by a large septal defect, which is both atrial and ventricular. The atrial septal defect is a patent foramen primum. This patency varies from a relatively small defect to almost complete absence of the posterior portion of the distal atrial septum. In many instances there is a patent foramen ovale due to defective formation of either the limbus (septum secundum) or the septum primum. In some instances the atrial septal defect begins at the proximal boundary of the limbus, so that the defect is a combined foramen ovale and foramen primum (cor triloculare biventriculare). The ventricular septal defect consists of an absence of the pars membranacea and the adjacent portion of the posterior ventricular septum. In a few instances the posterior ventricular septum may be markedly defective. The anterior ventricular septum is usually intact, although occasionally it may also be slightly defective.

The atrial septum in some cases is pushed a considerable distance to the left of the posterior interventricular sulcus. Where there is a markedly defective atrial septum, the coronary sinus may be duplicated and small. The right atrium and ventricle are always hypertrophied and dilated, with considerable endocardial hypertrophy and sclerosis. The left atrium is always small. The left ventricle is usually equal in size to that of the right, and its wall about equals the right in thickness.

Rogers and Edwards[32] previously emphasized the similarity between common atrioventricular orifice and patent foramen primum. However, we believe it is better to keep the distinction between the two anomalies, however slight be their morphologic differences in some cases, since the latter commonly permits survival to adult life, whereas the former is usually associated with death in the first decade.

Analysis of our four cases shows that the conduction system in common atrioventricular orifice presents a fairly common pattern (Fig. 12). The SA node presents

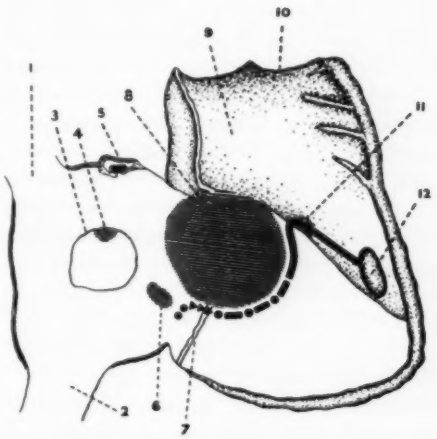


Fig. 12.—Diagrammatic sketch of the course of the AV node, bundle, and right bundle branch in the anomaly common atrioventricular orifice. Right atrial and ventricular view. Dots indicate AV node; triangles, penetrating portion of the AV bundle; dots and dashes, branching portion of the atrioventricular bundle; solid line, right bundle branch; 1, superior vena cava; 2, inferior vena cava; 3, limbus; 4, patent foramen ovale; 5, cut edge of atrial appendage; 6, entry of coronary sinus; 7, base of tricuspid valve; 8, combined patency of atrial and ventricular septa; 9, conus; 10, base of pulmonary valve; 11, muscle of Lancisi; 12, cut edge of moderator band.

no change. The AV node originates in the myocardium of the distal portion of the posterior wall of the right atrium and the adjacent portion of the inferodistal part of the atrial septum. This piece of septum, which lies below the foramen primum between the insertions of the inferior annulus of the mitral and the tricuspid valve segments to the posterior part of the central fibrous body, may be called the atrioventricular septum, separating right atrium from left ventricle. Where this septum is absent, or markedly displaced to the left, then the AV node arises from the posterior wall alone. The node lies distal to the mouth of the coronary sinus and proximal to the right atrioventricular sulcus. It ascends vertically or obliquely, penetrating the posterior part of the central fibrous body to become the atrioventricular bundle. (In the anomaly common atrioventricular orifice, there is no true central fibrous body but an anterior part to which is attached the

arterial trunks and the annulus of the anterior aspects of the atrioventricular valve and a posterior part to which is attached the annulus of the posterior aspects of the atrioventricular valve.) The bundle thus reaches the posterior rim of the combined septal defect distal to the atrial portion of the foramen primum, where it lies adjacent to the attachment of the mitral component of the common valve. (Sometimes it reaches the defect at the level of the common atrioventricular orifice.) The atrioventricular bundle now turns horizontally, skirting the combined septal defect. It first lies in a remnant of the pars membranacea atrioventriculare, adjacent to the atrioventricular portion of the foramen primum. In this manner it reaches the attachment of the annulus of the tricuspid portion of the common valve to the remnant of the pars membranacea. At this level the bundle is now related to the common atrioventricular orifice. It then passes through the remnant of the pars membranacea interventriculare and is now related to the proximal part of the interventricular foramen. It then comes to lie directly adjacent to the interventricular foramen as it sits on the summit of the posterior ventricular septum. Paraspecific fibers are given off from the junction of the AV node and atrioventricular bundle and from the latter as it passes through the posterior component of the central fibrous body and the pars membranacea atrioventriculare. As the bundle reaches the level of the common atrioventricular orifice, fibers of the posterior radiation of the left bundle branch begin to be given off. As the bundle reaches the distal wall of the combined defect, it divides into the right bundle branch and the remainder of the left. This remainder consists of some fasciculi going toward the apex of the heart and some to the anterior papillary muscle. The right may be made up of a single bundle, or numerous fasciculi may be given off at different levels which journey independently upward and distally to reach the lower margin of the crista. In the beginning of its course, the right may give off branches directly to the septum. Along the crista, the fasciculi of the right combine to form a bundle which proceeds to the moderator band and parietal wall.

Thus, when compared to the normal course of the conduction system, the following deviations are present in common atrioventricular orifice: 1. The AV node is displaced posteriorly, originating more from the posterior wall of the atrium. 2. The penetrating portion of the atrioventricular bundle passes through the posterior part of the central fibrous body instead of the normal central fibrous body, which is not formed in this anomaly. 3. In the absence of a well-formed atrial and ventricular septum, the atrioventricular bundle is displaced posteriorly, skirting the lower margin of the defect. 4. The right bundle branch may be given off in several rather than in a single trunk. 5. The posterior radiation going to the posterior papillary muscle is relatively small, while the main left is given off at the bifurcation going to the anterior papillary muscle and to the ventricular septal wall. 6. The right bundle branch may give off high paraspecific connections to the septum, which does not occur in the normal heart.[26]

The above abnormalities are explicable in the light of the embryogenesis of the conduction system.[34-38] It is the present consensus that the AV node and bundle in the mammal originates from the posterior part of the atrial canal musculature, which lies behind the posterior endocardial cushion, at a time when the musculature of this canal is still unbroken. The primordia of the structures appear just before or at a time when the inferior ventricular septum has joined the posterior endocardial cushion and before the endocardial cushions have fused. In man this occurs at about 8 to 10 mm. There is a difference of opinion as to whether the AV node and bundle originate in situ[36,37] or whether the bundle originates from a proliferation of atrioventricular nodal tissue.[38] Just before or at

the time the endocardial cushions are fusing (10 to 11 mm. in man), the beginning of the left bundle branch is seen. After the endocardial cushions have fused, the right bundle branch makes its appearance (at about 13 mm. in man). At this time the left bundle branch is of considerable size. When the interventricular foramen is becoming closed off (about 15 to 20 mm. in man), the left bundle branch is completely formed (16.5 mm.), and after the completion of the pars membranacea, the right bundle branch is traced to the moderator band (22 to 25 mm.). There is also a difference of opinion as to whether the bundle branches originate in situ from the ventricular trabeculae[36,37] or whether they originate from a proliferation of tissue of the atrioventricular bundle.[38] The definitive positions of the AV node in the lower part of the atrial septum and of the atrioventricular bundle in the lower part of the central fibrous body are secondary to the fusion of the endocardial cushions and subsequent closure of the foramen primum.

It has been accepted that common atrioventricular orifice is best explained as a lack of fusion of the dorsal and ventral endocardial cushions. This fused mass of endocardial tissue should send up a projection to the septum primum and secundum, thus closing the foramen primum. This fails to occur. Likewise, the endocardial cushions should be important participants in the foramation of the pars membranacea and the final closure of the interventricular foramen. This likewise fails to occur. Hence, there ensues a common atrioventricular orifice with a concomitant patent foramen primum and foramen interventriculare.

Since the AV node and bundle are developed posterior to the posterior endocardial cushion, when there is a lack of proliferation and fusion of this cushion with the anterior endocardial cushion, there naturally ensues a retention of the primitive state and a definitive posterior displacement of these structures. However, at all times the bundle keeps the primitive relationship to the posterior endocardial cushion (posterior part of the central fibrous body) and the summit of the posterior septum. Hence it always skirts the posterior rim of the defect, or, in maldevelopment of the posterior septum, it lies on the remnant of this septum. The right and left bundle branches which are developed later than the atrioventricular bundle are affected by the change in trabecular structure and hence produce the abnormalities in these branches mentioned above.

It is to be anticipated that this anomaly and others of its type may at times show disruption in continuity of the AV node, bundle, and bundle branches, as shown in the cases of Mönckeberg[3]; Yater, Barrier, and McNabb,[11] Yater, Leaman, and Cornell,[12] Wallgren and Winblad,[13] and Turpin, Lenègre, and Chassagne.[18] In these cases it may be postulated that hemodynamic sclerotic changes around the defect may secondarily affect these structures, either impeding their growth or disrupting their continuity.

Summary and Conclusions

The conduction system of four specimens, the seat of common atrioventricular orifice, was studied histologically.

The following abnormalities were found: (*a*) The AV node is displaced posteriorly. (*b*) The bundle of His skirts the lower margin of the defect. (*c*) The right bundle branch often arises from several separate fasciculi. (*d*) The right bundle branch may give off a high twig directly to the septum. (*e*) The left bundle branch has a short posterior radiation, most of the fibers emanating from the region of the bifurcation.

The changes in the position and course of the conduction system are related to the embryogenesis of the anomaly, common atrioventricular orifice, and the concomitant minor changes in the embryogenesis of the conduction system.

Mr. Frank Vinton provided technical assistance.

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Nasopharyngeal Angiofibromas

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Juvenile nasopharyngeal fibroma, nasopharyngeal fibroma, or angiofibroma is a comparatively uncommon benign neoplasm arising in the nasopharynx of adolescent males. Although histologically considered benign, it has been described as "clinically malignant" because of its expansive growth, infiltrative tendencies, and resistance to treatment. Scattered case reports and a few large but not too carefully selected series have appeared in the literature over the past few decades. Most of the articles have dealt with the clinical recognition and treatment of these lesions. It has been only within the past few years that comprehensive morphologic descriptions [1,2] have become available. Sternberg [1] described in detail the histologic features of the tumor and the wide variation in its appearance. He also correlated the clinical impression of retarded sexual development with the occurrence of the tumors in his group of patients. Martin, Ehrlich, and Abels [2] earlier had recognized evidence of delayed puberty in a clinical report of most of the same group of patients studied by Sternberg. Redoglia [3] reported a case in a 17-year-old boy whom he considered to be eunuchoid in physique. Kerwin [4] reported a case in a 16-year-old boy who was treated with androgens and radiation with good results. Kerwin also discussed the role of estrogens in the production of fibrous tumors experimentally. Martin et al.[2] discussed the effect of estrogens on blood vessels and of hormone therapy in the treatment of these tumors. Hemley, Schwinger, and Friedman [5] reported three cases but found no evidence of sexual or other immaturity in their patients.

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The older articles, such as the large series of 58 cases reported by Shaheen,[6] and Figi,[7] who reviewed 45 cases, included females. Doubt has arisen that such tumors do exist in the female. Finerman [8] described a nasopharyngeal angiofibroma in a 13-year-old girl. Sternberg [1] reviewed the slides from Finerman's case and felt that the lesion probably was not an angiofibroma but was an inflammatory nasal polyp.

The tissue from which the tumor arises has been the subject of some discussion. Ringertz [9] described the site of origin as the ventral periosteum of the bones which originate in the embryonal occipital plate. He accounted for new-bone formation which is occasionally seen in these tumors as evidence for this origin. Friedberg [10] agreed with this site and elaborated upon it. Brunner [11] described in some detail the origin as the fascia basalis, which he emphasized is distinct from the periosteum and composed of the pharyngeal aponeurosis and buccopharyngeal fascia. He also noted the usual occurrence of large vessels in the midline of the nose and nasopharynx in male infants. Brunner logically assumed that the tumors developed on the inner side of the superior constrictor muscle, as they never were covered by muscle. He felt that the angiofibroma could be multicentric in origin and that recurrence was possible even after complete removal.

Materials and Methods

Slides and paraffin blocks were available from all cases. The tissue had been formalin fixed, and sections were stained routinely with hematoxylin and eosin. Additional slides were prepared from the original blocks when indicated. In addition to the routine stains, sections from selected blocks were stained by Wilder's reticulum technique, with Masson's trichrome stain, and with a modified Verhoeff's elastic stain counterstained by Van Gie-

Clinical Abstract of Cases

Case No.	Age *	Sex	Symptoms	Duration of Symptoms	Physical Examinations †	Treatment	Total Duration of Disease	No. of Recurrences	Follow-Up
1	12	M	Nasal obstruction	10 mo.	N-P tumor	Surgical	10 mo.	0	Living & well, 30 yr.
2	9	M	Nasal obstruction; pain	3 mo.	Tumor of right naris	Surgical, x-ray, & radium	4 yr. 8 mo.	5	Living & well, 24 yr.
3	22	M	Nasal obstruction	5 yr.	N-P mass	Surgical	5 yr.	0	Died 10 mo. postoperatively from "throat hemorrhage"
4	19	M	Nasal obstruction	?	?	Surgical	6+ mo.	3	Living & well, 27 yr.
5	13	M	Epistaxis; discharge	Mo.	N-P mass	Surgical & radon	1 yr. 5+ mo.	2	Living & well, 24 yr.
6	17	M	Nasal obstruction; epistaxis	1 yr.	N-P mass, obstruction in right naris	Surgical, radon, & radium	4 yr. 6 mo.	3	Living & well, 23 yr.
7	17	M	Nasal obstruction	Mo.	Mass in epipharynx	Surgical & radium	2 yr. 6 mo.	5	Living & well, 23 yr.
8	17	M	Nasal obstruction	Mo.	N-P mass	Surgical & radium	Mo.	0	Living & well, 24 yr.
9	13	M	Nasal obstruction	Mo.	N-P mass	Surgical	Mo.	0	Living & well, 23 yr.
10	50	M	Mass of left face; left eye blind	Since age 13, off and on	N-P mass in antrum & sphenoid sinuses	Surgical & x-ray	37 yr.	12+	Died 12 mo. postoperatively from "paralysis of larynx & pharynx"
11	19	M	Nasal obstruction	8 mo.	Tumor in N-P	Surgical	2 yr.	0	Living & well, 20 yr.
12	16	M	Daily epistaxis; difficulty breathing	8 mo.	Mass in left naris & N-P	Surgical & x-ray	11 yr. 10 mo.	3	Living & well, 8 yr.
13	18	M	Nasal obstruction; epistaxis	1 yr.	N-P tumor	X-ray followed by surgical	1 yr.	0	Living & well, 16 yr.
14	14	M	Nasal obstruction	3 mo.	N-P tumor	Surgical	3 mo.	0	Living & well, 17 yr.
15	18	M	Nasal obstruction	3 yr.	"Frog face," N-P mass	Surgical & radium	3 yr.	5	Living & well, 16 yr.
16	14	M	Nasal obstruction; epistaxis	2 yr.	N-P mass with extention into each meatus	Surgical & x-ray	3 yr.	0	Living & well, 15 yr.
17	16	M	Epistaxis; nasal obstruction	3–4 yr.	Mass in left naris	Surgical	3–4 yr.	0	Living & well, 15 yr.
18	13	M	Nasal obstruction; swelling of left cheek; epistaxis	1 yr.	Mass in left naris	Surgical	1 yr.	0	Living & well, 14 yr.
19	12	M	Nasal obstruction; epistaxis	6 mo.	N-P mass, destruction of left antral wall	Surgical, x-ray, & radium	1 yr. 8 mo.	2	Living & well, 13 yr.
20	17	M	Nasal obstruction	1 yr.	N-P mass in right naris & cheek	Surgical, radium before surgery	1 yr. 6 mo.	1	Living & well, 11 yr.
21	11	M	Nasal obstruction; epistaxis	1 yr.	Mass in right naris & N-P	Surgical	1 yr.	0	Living & well, 13 yr.
22	27	M	Nasal obstruction; epistaxis	? yr.	Tumor N-P, obstruction choanae	Surgical	? yr.	0	Died; suicide
23	17	M	Nasal obstruction; epistaxis	10 mo.	N-P tumor projecting into nose	Surgical	10 mo.	0	Living & well, 12 yr.
24	18	M	Nasal obstruction; epistaxis	7 mo.	N-P mass occluding both sides	Surgical	4 yr. 6 mo.	3	Living & well, 5 yr.
25	14	M	Nasal obstruction	1 yr.	N-P mass, extention to left orbit	Surgical & x-ray	1 yr.	0	Living & well, 9 yr.
26	17	M	Nasal obstruction; epistaxis	9 mo.	N-P mass	Surgical	9 mo.	0	Living & well, 7 yr.

* Age when first examined.
† N-P indicates nasopharyngeal.

son's method. For the study of mast cells, appropriate sections were stained by the Giemsa method.

This series consisted of cases treated at the University of Michigan Hospital during the 25-year period from 1925 through 1949. During this period 26 cases of nasopharyngeal angiofibromas were treated (Table). There were no females in this group; all patients were white males. The age range was from 9 to 50 years. Excluding the one case who was 50 years old, the average age was 16 years.

When first examined, 22 patients were in the second decade (84.6%), 2 patients were in the third decade (7.7%), and 1 patient each was in the first and the sixth decades (3.8% each). Symptoms had been present before initial examination for 3 months to 5 years, with an average of about 15 months. Fourteen patients gave histories of symptoms for one to five years before seeking medical advice. The duration of disease varied considerably from an extreme of 37 years (Case 10) to 3 months (Case 14). The average duration of disease was approximately three years.

Eleven patients had one recurrence or more (42.3%), with an average of about three recurrences per case. The number of recurrences in these 11 patients varied from 1 to 5. Case 12 had a surgical procedure for a third recurrence 11 years 2 months after a second recurrent tumor had been excised. Fifteen patients (57.6%) did not have recurrence after the initial treatment.

Treatment consisted of surgical procedures and radiation, the latter either x-ray or interstitial radiation by radium or radon implants. Twelve

patients received combined treatment, consisting of a surgical procedure and irradiation. In 10 of the 12 patients, the surgical procedure was followed by radiation. Two patients, Cases 12 and 13, were treated initially by x-radiation, 5 months and 1 month, respectively, before undergoing surgical therapy. Of the 11 patients who developed recurrences, 9 were treated by a surgical procedure and radiation and 2 by operation only. Of the 15 patients who did not develop recurrences, 4 were treated by operation and radiation and 8, by operation only. No patient in this series was treated with androgens, either alone or in combination with other types of therapy.

Follow-ups were successfully accomplished on all patients. Three patients were reported dead; one from a "throat hemorrhage" 10 months postoperatively (Case 3), one from "paralysis of the larynx and pharynx" 12 months postoperatively (Case 10), and the third by suicide (Case 22). No necropsies were performed. The longest follow-up period was 30 years (Case 1), and the shortest period after the last operation was 5 years (Case 24). The study was concluded with the 1949 cases, so that at least five years would have elapsed since the last patient included in the series was treated.

Case 20 had a thyroidectomy for carcinoma five years after surgical removal of a recurrent nasopharyngeal angiofibroma, and two years after the thyroidectomy he had the removal of an osteoma in the left auditory canal. When last examined, in 1954, there was no evidence of recurrence of any of the neoplasms.

Symptoms most commonly recorded in the clinical records were nasal obstruction and epistaxis. The obstruction usually was unilateral, although instances of bilateral obstruction were not uncommon. Epistaxis occurred occasionally as a daily event. Only one patient, Case 15, had been described as presenting with a "frog face" type of deformity due to growth of the tumor into antra and orbits.

Gross Description

Grossly, the nasopharyngeal angiofibroma is a firm to resilient tumor of polypoid appearance. The color is gray to pink-gray, depending on the degree of squamous metaplasia of the mucosal surface. Ulcers covered by a purulent exudate are not uncommon. On section the tumor is pink to pink-gray in color and, if extremely vascular, may have a spongy or reticulated appearance on the cut surface.

Fig. 1 (Case 23).—Numerous thick-walled veins in the deeper portion of the tumor. Hematoxylin and eosin; ×60.

Microscopic Description

The vascular network and supporting stroma so well described by Sternberg [1] were apparent in all sections. In all such tumors there was an admixture of these two features. In the younger rapidly growing lesions the vascular pattern was outstanding; but in the older lesions the fibrous stroma dominated the picture. There was a wide variation in the vascular pattern in different tumors and even within the same neoplasm. However, basically, large and small thin-walled vessels were seen, separated by a variable amount of stroma. Vessels with moderately well-developed walls appeared in the deeper portions of the growth (Fig. 1). The endothelial cells of the medium and large-sized vessels were flattened and thin, while in the smaller more actively growing vessels the endothelial cells were plump and hypertrophic. A gradual diminution in the caliber of the vessels was noted as the mucosal surface was approached, until the pattern suggested a sclerosing hemangioma (Fig. 2). Occasionally proliferating angioblasts beneath the mucosa appeared as pseudorosettes or multinucleated giant cells, with the nuclei indistinguishable from those in the surrounding stroma (Figs. 3 and 4). In the older tumors, where there was a gradual compression of the vessels, the endothelial nuclei appeared as parallel rows of cells. Recent and older organized thrombi frequently were seen. No vasculitis was observed in this series, although Sternberg [1] described such changes.

The stroma was composed of coarse bundles of hyaline collagen in which variable numbers of stromal cells were dispersed. The collagen was arranged in interlacing bundles or in parallel wavy bands, with nuclei lined up at regular intervals along the bands suggesting fascia (Fig. 5). An occasional nerve was surrounded by tumor; spicules of bone were rarely encountered.

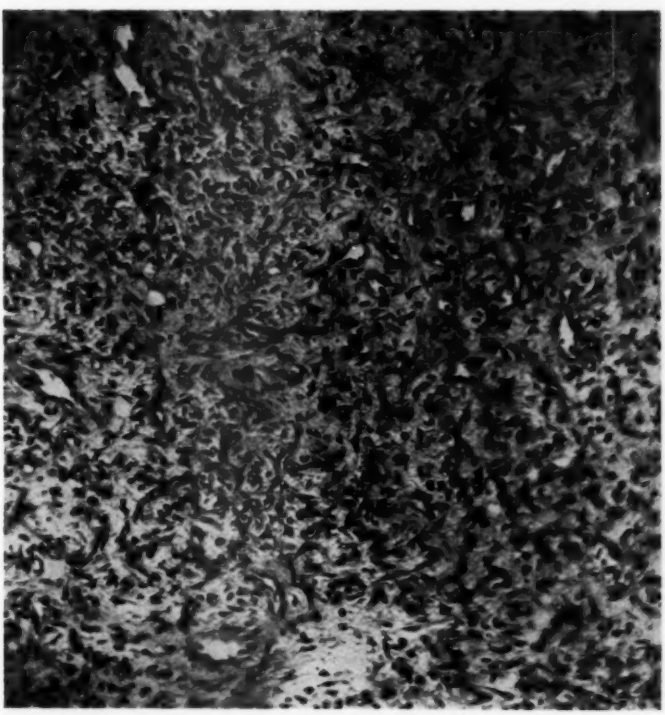

Fig. 2 (Case 7).—Proliferating capillaries resembling a sclerosing hemangioma. Hematoxylin and eosin; reduced 17% from mag. × 90.

Fig. 3 (Case 8).—Proliferating angioblasts beneath the mucosa (not shown). Grouping of the nuclei in the upper right and lower left corners of the field suggest multinucleated giant cells. Hematoxylin and eosin; reduced 8% from mag. × 160.

and no chondromatous foci were observed in the stroma. A myxomatous type of stromal degeneration was not infrequent, but usually in a restricted area, and never diffuse as seen in inflammatory polypi. In an occasional tumor there was an imperceptible blending of submucosal stroma, typical of an inflammatory polyp, into the dense cellular fibrous stroma so characteristic of the angiofibroma (Fig. 6). The coarse collagenous stroma occasionally persisted up to the mucosa, where it surrounded the submucosal glands. Capsules were not found, although in one patient, Case 11, part of the angiofibroma was enclosed by a capsule-like band of dense hyaline connective tissue which separated the tumor from the overlying mucosa and submucosa (Fig. 7).

The nuclei of the stromal cells were round to oval in shape, with vesicular pattern and a prominent amphophilic nucleolus; occasionally two nucleoli were present, and the nuclear membrane was usually distinct and sharply outlined. Although the nuclei have been described as stellate, it may be the cell outline which assumes this configuration. This was best seen in sections where the stroma had retracted from the cell outline during the prepartion of the section (Figs. 8 and 9). It was evident that the cytoplasm had various shapes, the commonest being stellate. There was also a tendency for the stromal cells to radiate

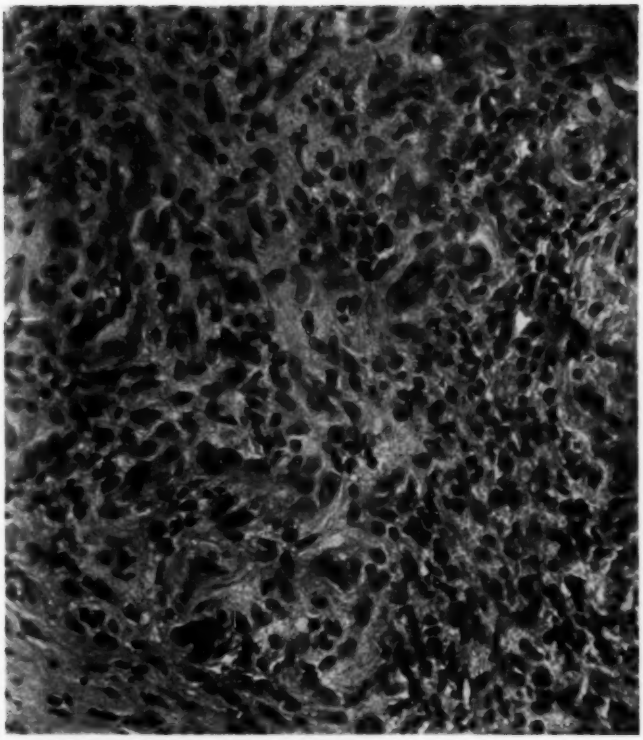

Fig. 4 (Case 17).—Plump nuclei of stromal cells, showing groupings suggesting giant cells, as in Figure 3. Slight leukocytic infiltration is evident. Hematoxylin and eosin; × 120.

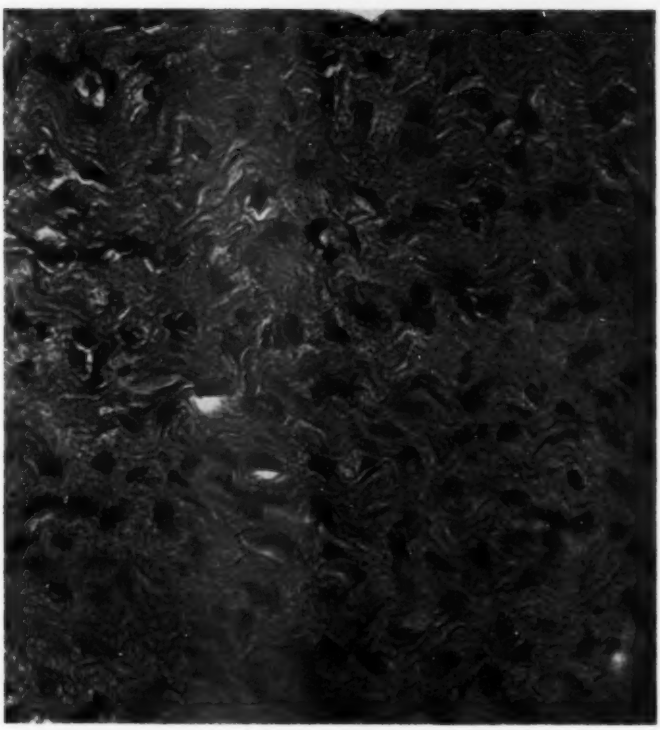

Fig. 5 (Case 3).—Parallel rows of cells and coarse collagen bundles suggesting fascia. Hematoxylin and eosin; reduced 15% from mag. × 160.

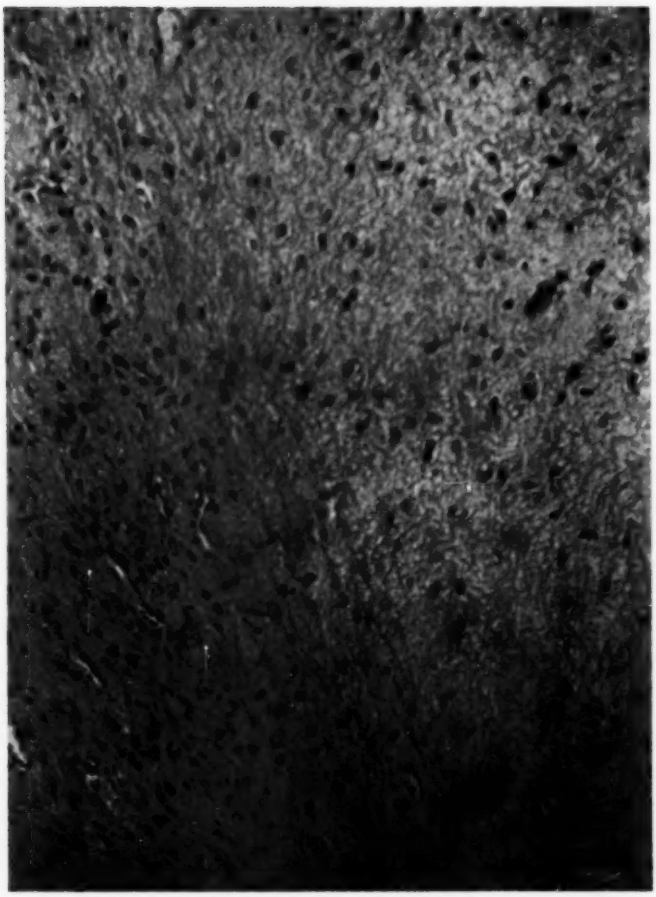

Fig. 6 (Case 19).—Blending of loose edematous stroma above into dense cellular fibrous stroma of angiofibroma below. Hematoxylin and eosin; reduced 15% from mag. × 90.

from the vessels [1]; not infrequently two nuclei were observed within a cell, and mitoses were rare.

Reticulum stains showed a diffuse reticulum network, each cell being surrounded by fused bands of fibers; elastic-fiber stains disclosed no evidence of elastic fibers, and no smooth-muscle fibers were observed in trichrome-stained sections.

A comparison of the histologic structures of angiofibromas from the younger patients in this series with those from the older patients revealed no essential differences in morphology. Apparently the duration of disease influenced the histologic features more than the age of the patient. Most of the patients in their late teens and early twenties gave a history of longer duration of symptoms than did those in the early teens; accordingly the tumors from patients with a history of symptoms for several months to years showed less vascularity and cellularity than did the tumors from patients with only a few months' history of symptoms. Recurrent tumors showed a more sclerotic pattern, in spite of the fact that they were actively growing. Tissue removed after irradiation tended to show more sclerosis. It was felt that in a few cases there were somewhat more bizarre cells and giant forms of stromal cells following irradiation.

The mucosa over the surface of these tumors may be of respiratory type; but it

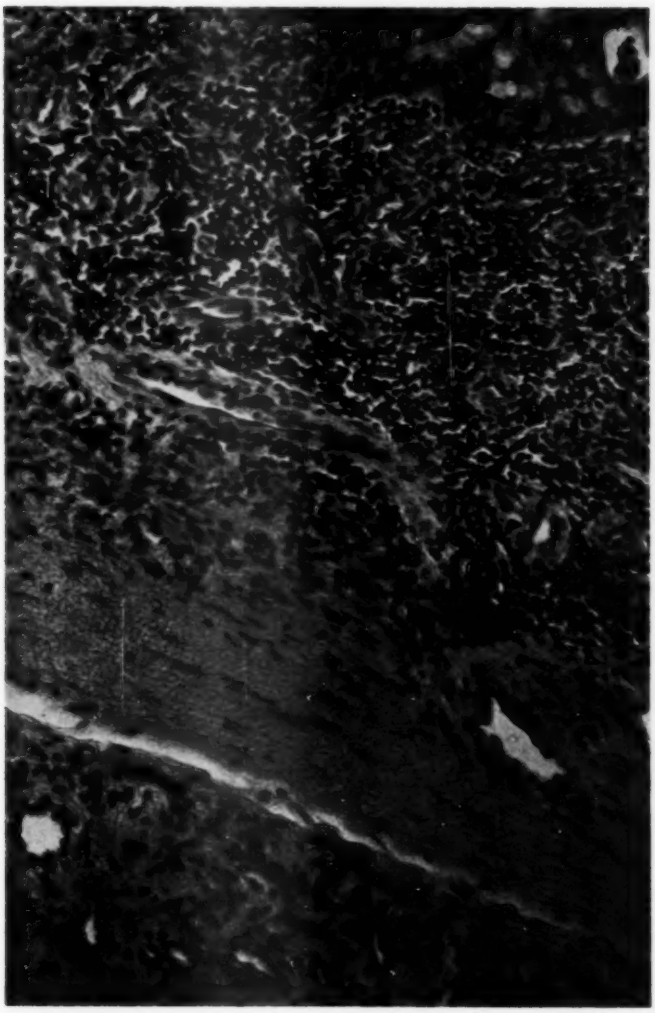

Fig. 7 (Case 11).—Pseudocapsule separating the angiofibroma below from the overlying chronically inflamed mucosa and submucosa. Hematoxylin and eosin; × 120.

frequently showed metaplasia to stratified squamous type, occasionally with a prominent keratin layer. Ulcers were common.

Two cases in the series deserve special comment, Cases 10 and 23. Patient 10 was a 50-year-old man who first developed symptoms of nasal obstruction at the age of 13 years. During his early teens he had 11 or 12 surgical procedures and extensive radiation therapy. He had no further known trouble until, at the age of 48 years, he was injured and the left side of his face swelled to "about one-half the size of a grapefruit." Elevation of the left eye was noted, and examination showed the left eye to be blind. A malignant tumor of the left antrum was suspected, and conservative treatment was advised. After seven months the tumor had increased to twice the original size, and an operation was performed. A large neoplasm was found to infiltrate the sphenoid and maxillary sinuses. It appeared to originate in the nasopharynx. The patient died 12 months later of "paralysis

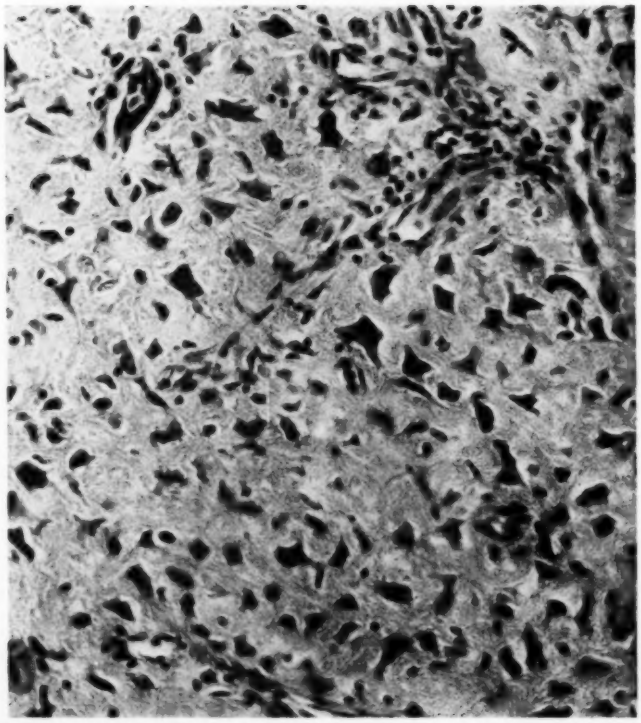

Fig. 8 (Case 23).—Bizarre multinucleated giant cells in stroma. Atypical cells were found throughout this tumor. There was no recurrence, and the patient was symptom-free 12 years later. Hematoxylin and eosin; × 120.

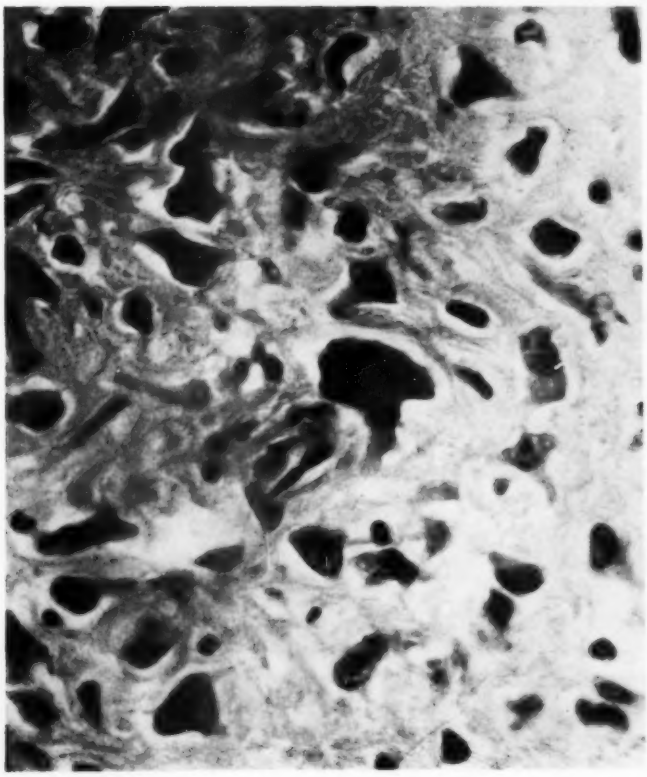

Fig. 9 (Case 23).—Higher power of a field from the same tumor seen in Figure 8. Hematoxylin and eosin; × 285.

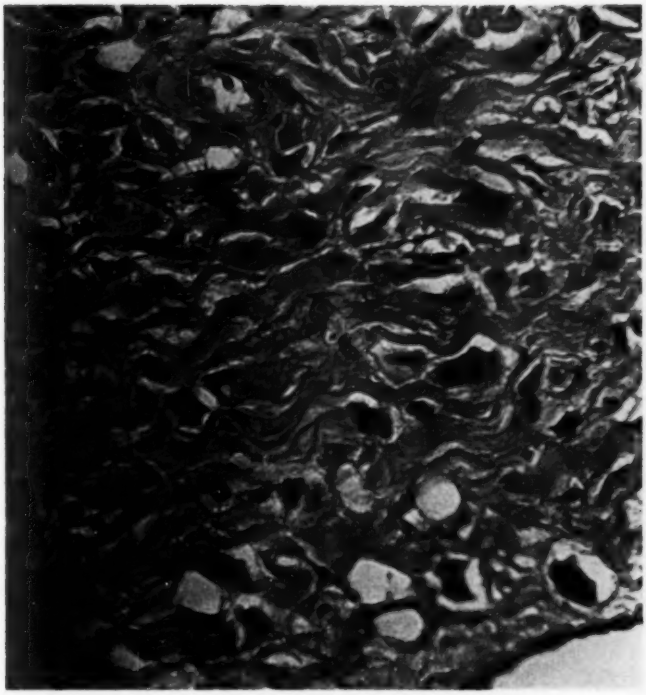

Fig. 10. (Case 10).—Tumor from a 50-year-old man. Very dense and somewhat atypical stromal cells. Hematoxylin and eosin; × 120.

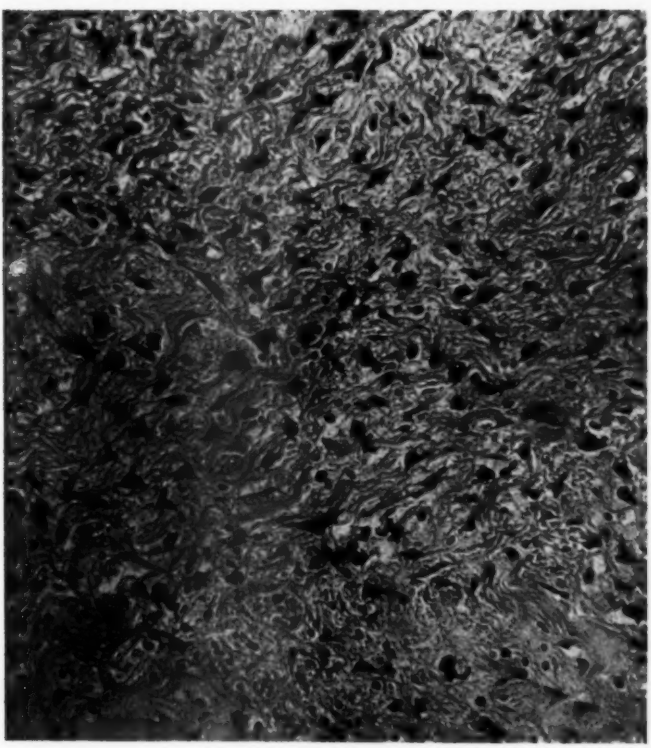

Fig. 11 (Case 6).—Numerous mast cells, seen here as dense round cells. Granularity is not apparent in the illustration. Giemsa stain; × 120.

of larynx and pharynx." Histologically there was no essential difference in the tumor from this patient and those seen in the younger age groups except marked sclerosis of the stroma (Fig. 10).

Patient 23 was a 17-year-old youth at the time of operation; he had a history of nasal obstruction and epistaxis for 10 months. At operation a large tumor was found arising in the nasopharynx and eroding into the ethmoid and sphenoid sinuses. There were no postoperative complications. Histologically the stromal cells in this tumor were the most unusual of the group and were bizarre in appearance, being multinucleated, hyperchromatic, and variable in size and shape (Figs. 8 and 9). Vascularity was only moderate in degree, and the atypical cells were separated by abundant hyaline collagen. The patient was alive, well, and without symptoms 12 years after operation.

An interesting finding in all the angiofibromas studied in this series was the presence of large numbers of mast cells. Selected sections of three tumors were stained by a modified Giemsa technique, and mast cells were counted by the method proposed by Janes and Johnson.[12] An average of eight mast cells per five high-power fields was found (Fig. 11). For comparison, sections of tonsils were stained with Giemsa stain, and an average of six mast cells per five high-power fields were found; these were in the connective-tissue septa of the tonsils. An inflammatory nasal polyp stained by the Giemsa method showed as many as 50 mast cells per high-power field. The presence of mast cells in these tumors apparently has no significance.

Differential Diagnoses

The diagnosis of nasopharyngeal angiofibroma should present no problem in the majority of cases. A clinical history of progressive nasal obstruction and epistaxis in an adolescent male who shows a rubbery pink to gray polypoid nasopharyngeal mass should engender suspicion.

Histologically the presence of the typical features of large thin-walled blood channels, dense collagenous stroma, and stellate fibroblasts confirm the diagnosis. A fibrosarcoma might be confused with an angiofibroma, but the latter neoplasm never exhibits the degree of cellularity seen in a sarcoma, and mitotic figures are difficult to find in angiofibromas. The occasional angiofibroma may have large bizarre multinucleated cells, as seen in Case 23 (Figs. 8 and 9); but the nuclei are widely separated by mature collagen, and mitoses are rare.

A superficial biopsy specimen may reveal only an edematous myxomatous stroma heavily infiltrated by plasma cells, lymphocytes, and eosinophils (Figs. 6 and 7). Such a specimen would not reveal the true neoplasm and might raise the question of an inflammatory nasal polyp. Such polypi may, if present for any length of time, develop a denser fibrous stroma than the usual myxomatous stroma seen in them; however, inflammatory nasal polypi do not contain the dense collagenous, almost fascial, stroma of the true angiofibroma. Inflammatory infiltrations are not ordinarily a feature of the angiofibroma. Unless secondarily infected through surface ulcers, only occasional wandering cells are seen.

The lesion which seems most apt to cause confusion is that of chronic productive inflammation. A small biopsy specimen may reveal only mature granulation tissue, which could suggest the stroma of an angiofibroma; however, the well-formed blood vessels and heavy inflammatory infiltrations are not seen in the angiofibroma unless there have been ulcers resulting in secondary infection.

Comment

The use of the adjective "juvenile" should be discarded in the description of this tumor. In addition to the 50-year-old patient in this series, Batsakis, Klopp, and Newman [13] reported a case in a 54-year-old man, and Martens and MacPherson [14] reported a case in a 55-year-old man. The

latter chose to classify the angiofibroma with true inflammatory hyperplasia. In the case reported by Batsakis et al.[13] a fibrosarcoma developed 10 months after partial surgical removal of an angiofibroma and intensive irradiation of the nasopharynx. At the time reported the patient had been symptom-free seven months after "total" surgical excision. Recently a recurrence of sarcoma has appeared locally, 14 months after excision.[15]

It is impossible in a small series of this size to state whether or not malignant transformation occurs in these tumors. The patient reported by Batsakis et al.[13] was a middle-aged man who received intensive irradiation following the original attempts at removal. The time interval between the irradiation and the appearance of the sarcoma seems to be too short for malignant transformation to have occurred as a result of the irradiation of the angiofibroma. It seems likely that a sarcoma was present from the beginning but was not present in the original surgical material. In the only case in this series which caused concern as to whether it was benign or malignant morphologically (Case 23, Figs. 8 and 9) the patient was alive and well 12 years after operation and without recurrence. None of the 13 patients in this series who received radiation as part of the treatment developed sarcoma.

Sternberg [1] emphasized the vascular and stromal elements in the angiofibroma of the nasopharynx and felt that the vascular network was an integral part of the tumor. His impression was that the "juvenile" nasopharyngeal angiofibroma was a distinctive type of hemangioma and in part behaved like the hemangiomas of childhood. It has been observed repeatedly that cutaneous hemangiomas of childhood tend to undergo spontaneous regression with age.[16] Apparently this neoplasm does not ordinarily regress spontaneously, or only those patients in whom the growth was so vigorous that it produced signs and symptoms were referred to this hospital. An unknown number of patients with the tumor, which is not observed because of its location, may have spontaneous regression of an undiagnosed neoplasm.

The nasopharyngeal angiofibroma may be considered to be a distinctive type of hemangioma seen predominately in adolescent males but having the tendency to persist until well past the age of puberty, as suggested by Sternberg.[1]

Summary

Twenty-six cases of nasopharyngeal angiofibroma collected from the material at the University of Michigan Hospital over a 25-year-period (1925-1949) are reviewed.

All patients were white males, with an average age of 16 years. One case occurred as a recurrence in a 50-year-old man.

Eleven of the twenty-six patients, or 42.3%, had one to five recurrences, with an average of three recurrences per case.

Nasopharyngeal angiofibroma may be a peculiar type of hemangioma, occurring almost exclusively in adolescent males but capable of persisting beyond adolescence. Malignant transformation probably does not occur.

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Invasiveness of Transplanted Tumors in Mouse Brain

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Introduction

Metastasis formation is dependent on many ill-understood phenomena, some of which have been studied from different approaches with use of transplantable tumors. It is the case, however, that the majority of available transplantable tumors rarely metastasize; this is so in spite of the fact that almost all these tumors are rapidly growing and appear histologically and cytologically malignant. It has been noted in our studies that transplantation to the brain affords an excellent technique for observing invasiveness, most easily seen in terms of invasion of the Virchow-Robin perivascular spaces. In the present study six different transplantable tumors of the mouse have been compared following transplantation into the brain of homologous hosts. Some of these tumors metastasize, others do not when transplanted subcutaneously. Their propensities for invasion of the perivascular spaces have been contrasted and correlated with their metastasizing capacity. It is believed that the technique of brain transplantation constitutes a particularly satisfactory approach to the study of the invasiveness of different tumors. This is more useful in view of the possibility of heterologous transplantation in this location.[1,2]

Material and Methods

The following mouse transplantable tumors were used:

1. Carcinoma T150 originated in the bladder epithelium of a C57 Black mouse. It is now an undifferentiated carcinoma that takes in 100% of cases, when transplanted subcutaneously. It metastasizes widely to the lung two weeks after implantation.[3]

2. Mammary adenocarcinoma H2712 originated in a C3H mouse and takes in 100% of the transplants.[4] No metastases were observed in animals inoculated with low doses of tumor suspension within 50 days.

3. Mammary adenocarcinoma C3HBA originated in a C3H mouse and takes in 100% of the transplants.[4] When transplanted subcutaneously it shows local invasion but seldom metastasizes to the lung.

4. Sarcoma T241 Lewis is derived from a carcinogen-induced fibrosarcoma in a C57 Black mouse.[4] It is now an indifferentiated pleomorphic-cell sarcoma with 100% of takes. It frequently metastasized to the lung.[6]

5. Fibrosarcoma GL46 is a carcinogen-induced fibrosarcoma originated in this laboratory in a DBA/2 mouse. When transplanted in the subcutaneous tissue, it takes in 100% of cases and does not metastasize, except when transplanted in the tail region.[6]

6. Amelanotic melanoma DBA/1 is a slow-growing tumor originated in a DBA/1 mouse, which has been found not to metastasize as long as 72 days after subcutaneous inoculation.

These six tumors were inoculated into related hosts as a cellular suspension prepared with one part of the tumor and five parts of saline. The suspension was obtained with a tissue grinder or by mincing the tumor with scissors. Before inoculation the mixture was passed through needles Nos. 18, 22, and 26. Penicillin was added to the tumor suspension, or the animals were given antibiotics after transplantation. The tumor suspensions were inoculated into the brain with a No. 26 needle with use of light ether anesthesia. The implant was made into the anterior segment of the left hemisphere through the left parietal bone, except in Experiments I(*b*) and V(*b*), in which it was made through the left orbital cavity. The dose of tumor suspension injected varied between 0.005 to 0.015 ml., according to the experiment. The animals were killed at various intervals after inoculation. Some of them died spontaneously before the fixed time and were disregarded. Generally the brain was removed from the calvarium and sections were taken parasagittally or

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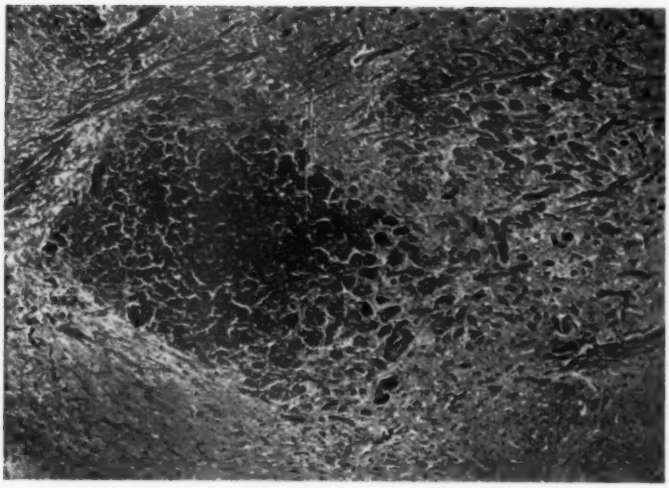

Fig. 1.—Carcinoma T 150; 11 days after transplant. Widespread around the main tumor growth; reduced 40% from mag. × 80.

coronally after formalin fixation, but in several cases sections were made through the entire skull after decalcification. All histological sections were stained with hemotoxylin and eosin.

Experiments and Results

EXPERIMENT I.—*Carcinoma T150 into C57BL/6 Mice*

(*a*) Forty male and female mice, 3 months old, were given injections of 0.01 ml. of tumor suspension into the brain through the parietal bone. Eight animals were killed after 3 days; ten, after 6 days; nine, after 9 days; 3 died spontaneously after 10 days, and 6 were killed 11 days after inoculation. Four animals were lost.

In the first group histological examination of several parasagittal sections of the brain revealed a small hemorrhagic focus surrounded by a few neoplastic cells in only three animals. In the second group the tumor was present in 9 animals out of 10 in the form of a small carcinomatous nodule surrounded by small nests of neoplastic cells invading perivascular spaces. In the remaining three groups tumor was present in seven out of nine mice killed after 9 days; in three out of three spontaneously dead after 10 days, and in six out of six

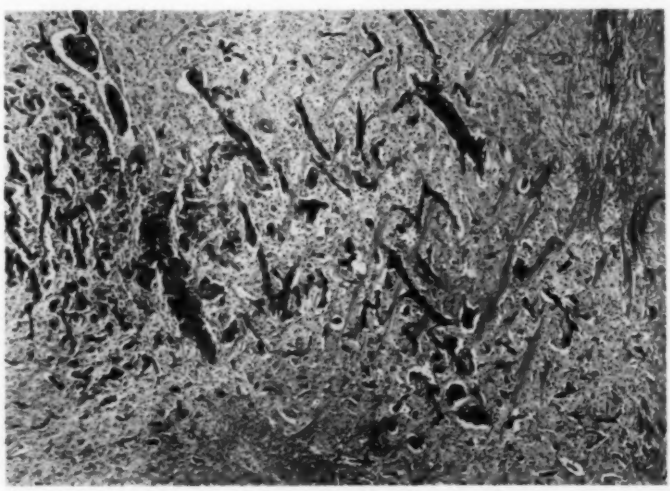

Fig. 2.—Carcinoma T 150; nine days after transplant. Invasion of the Virchow-Robin perivascular spaces; reduced 40% from mag. × 100.

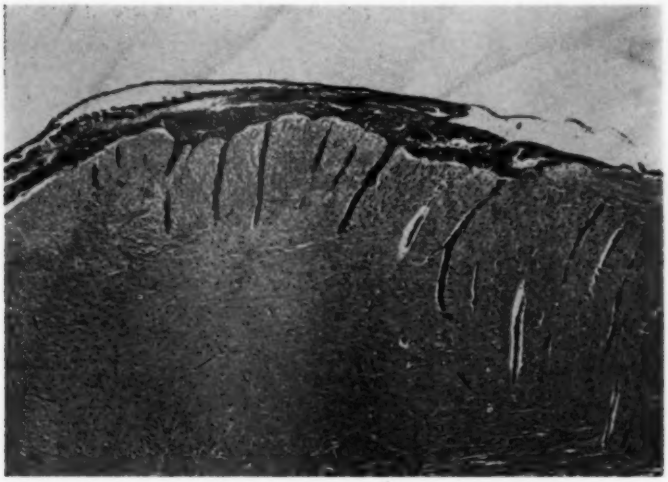

Fig. 3.—Carcinoma T 150; 11 days after transplant. Tumor growth in the meningeal spaces, with secondary invasion of the cerebral parenchyma through the perivascular spaces; reduced 40% from mag. × 80.

killed after 11 days. In these three groups microscopic study revealed the tumor to be very large, almost completely involving the anterior portion of the left hemisphere. The central area of the tumor was compact and very cellular and completely obliterated the nervous structures. The neoplastic growth had the features of an undifferentiated carcinoma: the cells and their nuclei were very pleomorphic, and numerous mitoses were present. There was very scant supporting stroma, and focal areas of necrosis and hemorrhage were seen. At its periphery the tumor was actively growing in multiple digitations, infiltrating the cerebral parenchyma with narrow strands of cells. In some instances the neoplastic proliferation seemed to penetrate the nervous structures directly, but in most cases it was possible to demonstrate the tumor spread along the perivascular spaces, surrounding dilated vessels. There was no evidence of vessel invasion. The carcinoma was also present in the meningeal spaces, particularly in the area between cerebellum and cerebral peduncle. In some instances the tumor in-

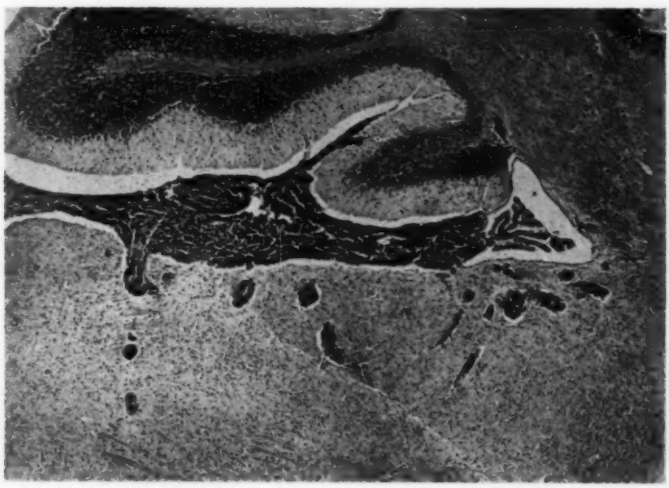

Fig. 4.—Same as Figure 3.

Fig. 5. — Mammary adenocarcinoma H2712; nine days after transplant. Tumor nests in the center of the hemisphere; reduced 40% from mag. × 80.

vaded the cerebral peduncle directly from the meninges, with narrow branches in the perivascular spaces.

(*b*) Twenty male mice, 4 to 6 months old, received an inoculation of 0.005 ml. of tumor suspension through the orbital cavity. Ten animals were killed 9 days after inoculation, and eight after 11 days. Two animals died before the fixed time and were discarded.

In all 18 animals the transplant took successfully. Microscopic study of parasagittal sections of the brain showed the carcinoma to be present around the route of penetration of the needle into the brain and in the center of the cerebral hemisphere, with very marked spread through invasion of perivascular spaces. Tumor growth was also present in the meningeal spaces from where invasion of the brain, especially in the region of the cerebral peduncle, had occurred. Section of the orbits showed neoplastic infiltration of the intraorbital tissues.

EXPERIMENT II.—*Mammary Adenocarcinoma H2712 into C3H Mice*

Twenty 2½-month-old female mice were given inoculations into the brain through the parietal bone of 0.01 ml. of tumor suspension. All animals were killed nine days after inoculation.

The transplant took successfully in 19 animals. Microscopic examination of para-

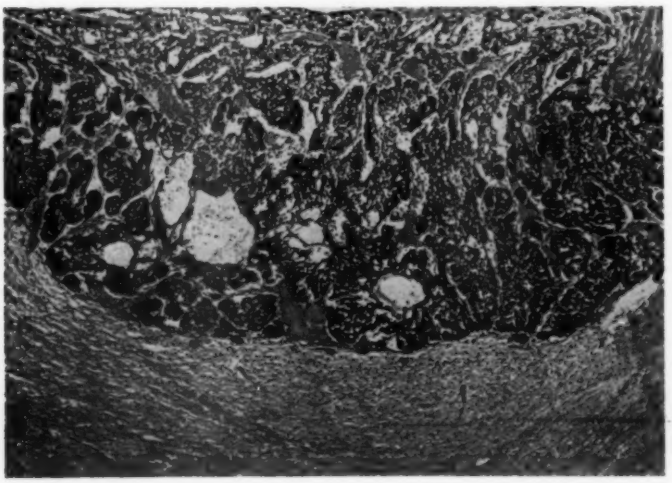

Fig. 6.—Same as Figure 5. Large tumor growth in the meningeal spaces without secondary invasion of the cerebral parenchyma; reduced 40% from mag. × 80.

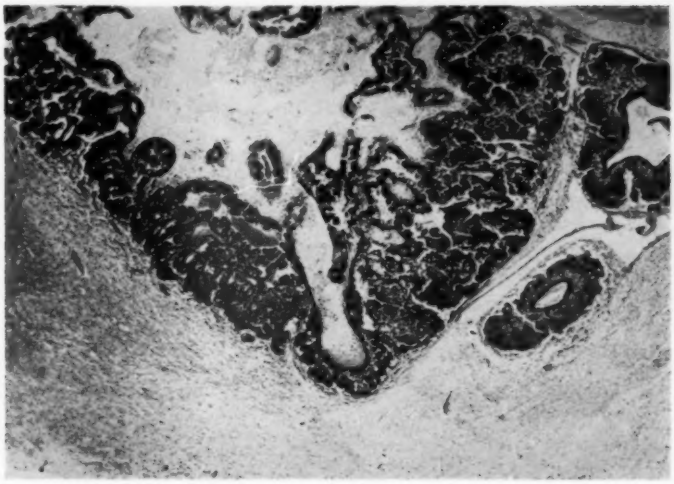

Fig. 7. — Mammary adenocarcinoma C3HBA; 18 days after transplant. Sharply delimited tumor; reduced 40% from mag. × 80.

sagittal sections showed several solid nests of neoplastic cells deeply in the brain in the area of inoculation. These nests were sharply demarcated. Although some of them were actually growing around vessels in the perivascular spaces, there was very scant spread around the inoculation site. Much tumor growth was also present in the meningeal spaces, particularly at the base of the brain. Around the tumor the cerebral parenchyma was compressed and presented focal areas of necrosis, but there was no secondary infiltration into the hemisphere. In both the intracerebral and the meningeal location the mammary adenocarcinoma was undifferentiated, showing only pseudoglandular structures. Cells and nuclei were very irregular in shape and size; nucleoli were prominent, and mitotic figures, frequent.

EXPERIMENT III.—*Mammary Adenocarcinoma C3HBA into C3H Mice*

Five 2½-month-old female mice were given injections into the brain through the parietal bone of 0.01 ml. of tumor suspension. They were killed 18 days after inoculation.

The transplant took in all five animals. Histological study of coronal sections in all

Fig. 8. — Sarcoma T 241; 11 days after transplant. Widespread, with infiltration of the perivascular spaces; reduced 40% from mag. × 80.

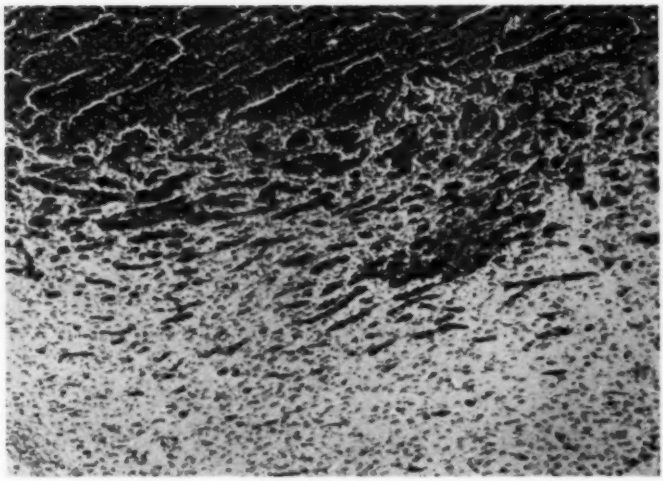

Fig. 9.—Same as Figure 8; reduced 40% from mag. × 140.

cases revealed a very large tumor growth in the middle of the cerebral hemisphere into which the suspension was injected. In the center the tumor presented areas of necrosis and cystic cavities. It was very cellular, tended to form pseudoglandular structures, and presented a high rate of mitosis. The neoplastic tissue compressed the surrounding cerebral parenchyma, but appeared to be well delimited. No perivascular invasion was seen.

EXPERIMENT IV.—*Sarcoma T241 into C57BL/6 Mice*

Ten 2-month-old female mice were given inoculations into the brain through the parietal bone of 0.01 ml. of tumor suspension. The animals were killed 11 days after inoculation.

In all of them the tumor grew successfully. Parasagittal sections showed a very pleomorphic and undifferentiated sarcoma growing in the cerebral parenchyma, with large and active spread in the perivascular spaces. The tumor was also present in the

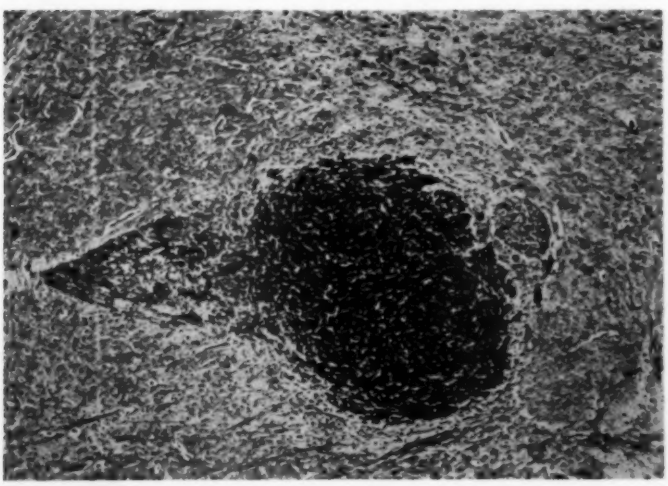

Fig. 10.—Fibrosarcoma GL46; nine days after transplant. Well-delimited tumor nodule in the center of the hemisphere; reduced 40% from mag. × 120.

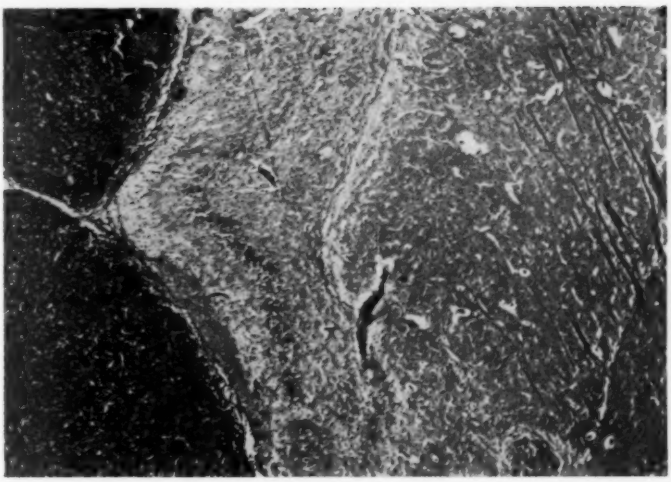

Fig. 11.—Fibrosarcoma GL46; 15 days after transplant. Large tumor growth in the meningeal spaces without invasion of the cerebral parenchyma; reduced 40% from mag. × 80.

meningeal spaces, from where it infiltrated the brain with narrow projections along the Virchow-Robin spaces. There was no evidence of vessel invasion.

EXPERIMENT V.—*Fibrosarcoma GL46 into DBA/2 Mice*

(*a*) Twenty-six 1½-month-old females were given injections into the brain through the parietal bone of 0.01 ml. of a suspension of fibrosarcoma GL46. The animals were killed nine days after inoculation.

The tumor took successfully in all the animals. Histological sections were performed parasagittally and showed the fibrosarcoma actively growing either in the meningeal spaces or in the cerebral parenchyma. It was formed by spindle-shaped or irregular cells with a certain amount of intercellular ground substance. Mitotic figures were prominent. In most cases the tumor was quite expansive and compressed the nervous structures with an evident boundary. In some instances there was a very moderate degree of spread, always through the perivascular spaces.

(*b*) Thirty 2-month-old female DBA/2 mice were given inoculations into the brain through the

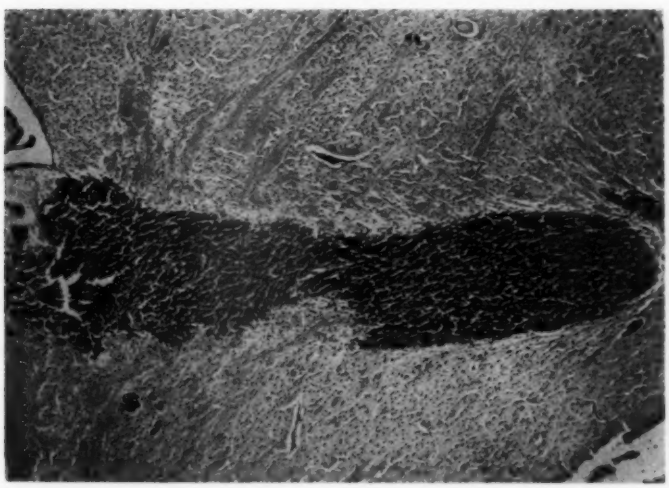

Fig. 12.—Fibrosarcoma GL46; 15 days after transplant. Sharply delimited growth, with very scant perivascular infiltration; reduced 40% from mag. × 100.

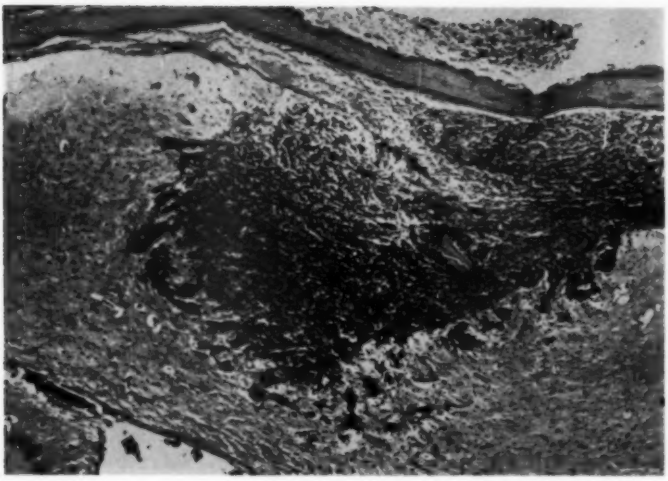

Fig. 13.—Fibrosarcoma GL46; nine days after transplant. Moderate spread around the site of inoculation; reduced 40% from mag. × 100.

orbital cavity of 0.005 ml. of tumor suspension. Ten animals were killed 12 days after inoculation, the remaining twenty, 15 days after.

Microscopic examination of parasagittal sections of the brain showed a pathological picture similar to that of experiment V(*a*). The tumor was present in all the animals and consisted of large compact nests. They were sharply circumscribed. There was very scant invasion in the Virchow-Robin spaces.

EXPERIMENT VI.—*Amelanotic Melanoma into DBA/1 Mice*

Twenty 9-week-old male and female mice were given inoculations into the brain through the parietal bone of 0.01 ml. of tumor suspension. Eleven animals were killed 9 days after inoculation, and nine, after 29 days.

Microscopic examination of parasagittal sections of the brain showed no evidence of tumor in the first group. In only two out of the nine mice of the second group was there a large tumor mass in the meningeal spaces. The tumor was composed of irregularly shaped cells with large nuclei and nucleoli and high mitotic ratio. The cytoplasm was abundant, and in a few cells it contained very evident melanin granules. In both cases the tumor growth was expan-

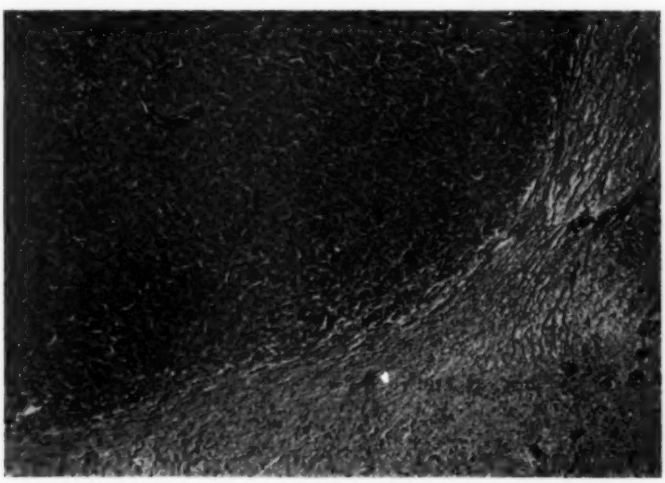

Fig. 14. — Amelanotic melanoma; 29 days after transplant. Large tumor sharply demarcated; reduced 40% from mag. × 100.

sive and compressed the cerebral structures. There was no evidence of secondary infiltration into the brain from the meningeal spaces. In one of the two cases a tumor nodule was also present in the center of the hemisphere at the site of the inoculation. At its periphery it showed a moderate degree of infiltration along the perivascular spaces. In the remaining seven mice the transplant did not take.

Conclusions

On the basis of histological and cytological characteristics all the six transplantable tumors used in this study were malignant. Only two of them, Carcinoma T150 and Sarcoma T241, metastasized regularly to the lungs following subcutaneous implantation. The other four tumors, in spite of their malignant characteristics, remain localized and seldom if ever give rise to metastatic growths. Five of these six tumors were found to grow well following intracerebral inoculation in homologous hosts. The sixth tumor, amelanotic melanoma of the DBA mouse, only took in 2 of 20 mice given injections. Technical difficulties, particularly the long latent period in the growth on transplantation of this tumor, must be blamed for this. In the two positive instances the tumor grew well, with numerous mitoses and no necrosis.

From the histological study of these tumors growing in brain substances it is clear that although all six neoplasms have histological and cytological characteristics of malignancy they expand in very different fashions. The Carcinoma T150 and Sarcoma T241 spread widely in the brain, mainly through invasion of the Virchow-Robin spaces; both these tumors additionally grew in the meningeal spaces, from where they secondarily invaded the cerebral parenchyma, again by infiltration of the perivascular spaces. There was no evidence of blood vessel invasion. The other four tumors remained well delimited although growing actively and destroying surrounding brain parenchyma by compression. This behavior on the part of these tumors is well correlated with their behavior as seen following subcutaneous transplantation; only T150 and T241 produce metastases regularly, and only these tumors grow in an invasive fashion in the intracerebral location. It is particularly interesting to note the intermediate behavior of GL46, which while growing generally by expansion did reveal a small degree of invasion of the perivascular spaces. Although this tumor does not metastasize following subcutaneous inoculation, it does spread widely following transplantation in the tail.[6]

It is of note that even the most invasive of these tumors did not appear to gain entry into the blood vessels. It seems clear that the anatomical arrangement of the vessels in the brain, surrounded as they are by the perivascular spaces, accounts largely for the lack of metastases from cerebral malignant neoplasms.

Summary

Experiments were undertaken in order to compare the pathological behavior of different tumors when transplanted into the brain of homologous mice. Six tumors were used. Two of them, Carcinoma T150 and Sarcoma T241 Lewis, that metastasize extensively when transplanted subcutaneously, showed widespread invasion of the Virchow-Robin perivascular spaces. The other four tumors, mammary adenocarcinoma H2712 and C3HBA, fibrosarcoma GL46, and amelanotic melanoma DBA/1, are tumors with no or very low metastasizing capacity when transplanted in the subcutaneous tissue. In the intracerebral transplantation they grew in well-delimited nodes; perivascular invasion was absent or very scarce. It is concluded that inoculation of transplantable tumors into the brain of homologous hosts is useful in order to study their invasiveness and spreading capacity.

Dr. Philippe Shubik gave many suggestions in this study. Photographs were taken by Mr. Thomas Scanlon.

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Correction

In the article "Atherosclerotic Lesions in Rabbits with Hemorrhagic Lipemia," by Drs. A. C. Ritchie, P. Macklem, and B. I. Weigensberg, in the November, 1957, issue of the ARCHIVES, the first two sentences of the paragraph beginning four lines from the end of page 556 should read as follows: "Fourteen small atherosclerotic lesions of the aorta were detected. They were equally common in the proximal and distal parts of the aorta but did tend to occur in the intimal mounds which stand beside the mouths of the arterial branches."

Influence of Denervated Muscles on Exostoses of Rats Fed a Sweet-Pea Diet

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Abnormalities of the skeleton, among them exostoses, have been observed to be prominent signs of the lathyrism-like disease produced in the rat by a diet which contains meal of the seeds of the sweet pea Lathyrus odoratus.[1-8] It has recently been shown that exostoses of this disease of the rat are formed at the point of attachment of muscles to the skeleton. Further, it has been demonstrated that one of the commonly occurring exostoses, namely, the pectineus-adductor longus exostosis, is formed by the tissues of the inner layer of the segment of the periosteum which serves for attachment of the related muscles.[9] It has also been shown that functional inactivation of muscles by transection prevents formation of the exostoses commonly produced at their skeletal attachments. Lastly, transection inactivation of one muscle or a number of muscles causes new exostoses to form at the attachment of muscles which assume the function of those transected.[10]

The observation that for Lathyrus-stimulated rats transection of muscles prevents formation of exostoses related to them, demonstrates that formation of exostoses by lathyric rats is dependent on and occurs only through the simultaneous stimulation of exostosis-producing tissues by two factors, namely, the Lathyrus factor and a muscle-tension factor. This observation appeared to us to be of sufficient significance to warrant confirmation studies. This observation also appeared to offer opportunities for investigation of exostosis patterns of lathyric rats and to provide a method for altering those patterns. This is a report of a confirmation study and a study of the exostosis patterns of the femur and innominate bone related to the adductor and flexor muscles of the thigh and extensor muscles of the leg innervated by branches of the lumbar plexus. The femur and innominate bone were selected for study because descriptions of some of the exostoses and muscles related to them have been presented in our earlier publications.[9,10] Because the small skeletal muscles of the rat are not readily transected and because transection manipulations may directly or indirectly damage exostosis-producing periosteal tissues, the method of functional inactivation of muscles by destruction of their nerve supply was selected for this study.

Methods

As a means of limiting, as far as possible, the problem of distinguishing between normally occurring developmental and growth changes of the skeleton and changes of the skeleton produced by the procedures of the experiment, only adult rats were used for this study of the influence of muscles on the origin and formation of exostoses.* Thirty-five female rats ranging to one year of age, with an average body weight of 249 gm. and a range in body weight of 203 to 314 gm., were subjected to the various experimental procedures described below.

To permit each animal to serve as its own control for the experimental procedures employed, the nerves to the muscles of only one posterior appendage were sectioned. Since plans included transection of elements of the lumbar plexus and since the liver, stomach, and spleen hamper opera-

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*Obtained from Holtzman Rat Company, Madison, Wis.

tions on the left dorsal abdominal wall, the right side was selected for nerve section for all animals. Nerves to be sectioned and the level at which the transection was to be performed were selected on the basis of exploratory dissections and information presented in publications on the anatomy of the rat.[11,12] All animals were prepared for operation in the usual manner, and the operations of nerve section were carried out with use of pentobarbital (Nembutal) anesthesia.

To determine the relative importance of the pectineus and adductor longus muscles in stimulating formation of the pectineus-adductor longus exostosis of the femur by lathyric rats, the nerve supply of the right adductor longus muscle was interrupted for some of our animals. That muscle of the rat is supplied by branches of the anterior division of the obturator nerve located internal to the pectineus and adductor longus muscles. To expose the nerve supply of the muscle, an incision extending from the inguinal ligament halfway to the knee was made through the skin of the medial surface of the right thigh; the skin was reflected, and the pectineus and adductor longus muscles were exposed and separated. The branching of the anterior division of the obturator nerve was found to be irregular, and as a result the anterior division, or its branches, were transected, whichever appeared most readily executed. Complete denervation of the adductor longus muscle was not obtained for all animals.

Through section of the trunk of the obturator nerve, some of our animals were to yield information on the influence of the adductor group of muscles on the various exostoses of the femur and innominate bone related to them. For those animals, the abdominal cavity was opened by a ½ in. or longer incision through the abdominal wall, placed to the right of the midline and ending near the inguinal ligament. The viscera were pushed to the left in order to expose the dorsal wall, and the obturator nerve was located by separating the connective tissues in the groove between the adductor caudae externus and internus muscles. The trunk of the right obturator nerve was cut at the posteromedial margin of the psoas major muscle.

Some of the animals of this experiment were to demonstrate the influence of the iliacus and psoas major muscles on the origin and differentiation of the iliacus-psoas major exostosis of the femur. Information on the innervation of those muscles is incomplete and unsatisfactory. However, the shape and position of its origins indicated that the psoas major muscle of the rat, like that of man, must be supplied by several nerves arising from the lumbar nerves and possibly also from elements of the lumbar plexus. It appeared that if denervation of all or the greater part of the psoas major muscle as well as the iliacus muscle was to be obtained, surgical interruption of the nerves of origin of the lumbar plexus was necessary. Therefore, for these animals, even though the fibers of the femoral and obturator nerves would also be destroyed, transection of the anterior divisions of lumbar Nerves 2, 3, and 4 was performed.

For the animals of the third part of this study, the abdominal cavity was opened and the right dorsal abdominal wall was exposed by the procedures described above for transection of the right obturator nerve. To locate the nerves to be transected, the obturator nerve was traced to its origin from the anterior divisions of lumbar Nerves 3 and 4 and the genitofemoral nerve was traced to its origin from the anterior division of lumbar Nerve 2. Transection of only the femoral nerve, at its origin from the lumbar plexus, was performed for one animal of this section of the study.

Following the operation of nerve section, the animals were placed in separate cages and fed a diet composed of equal parts of meal of Purina Laboratory Chow and meal of the seeds of the sweet pea Lathyrus odoratus. Because some exostoses of the femur of lathyric rats by growth and expansion become confluent and indistinctly separated from each other, the animals were killed after different periods on the lathyrogenic diet. For convenience in presentation, the number of animals, length of time fed the lathyrogenic diet, and major experimental procedures employed are summarized in the Table.

All animals were killed with ether, skinned, subjected to autopsy examination, and the carcasses, with posterior appendages in full abduction, were fixed and preserved in a 10% formalin solution. The muscles of the dorsal abdominal wall, the pelvis, and the thigh of the right side were examined for signs and degree of atrophy. The exostoses of the femur and innominate bone of

Summary of Animals Included in Experiment

Duration of Lathyrus Diet, Wk.	Animals, No.		
	Group 1 *	Group 2 †	Group 3 ‡
1	1	1	1
2	1	1	1
3	1	1	1
4	1	2	1
5	1	2	1
6	1 §	2	1
7	1 §	2	1
8	1	2	1
9		1	1 ‖
10		1	1
11		1	
12		1	
Total animals	8	17	10

* Nerve supply to adductor longus muscle interrupted.
† Obturator nerve transected at border of psoas major muscle.
‡ Nerves of origin of lumbar plexus transected.
§ Nerve to right adductor longus muscle intact.
‖ Femoral nerve sectioned at origin from lumbar plexus.

both right and left sides were exposed by careful dissection of the muscles of the thighs and pelvis. Verification of successful nerve transection was made during the dissection of the muscles and exostoses.

Results

The observations of this study of lathyrism in the rat are varied and numerous, but all results are related to the problem of the influence of muscles and groups of muscles on exostoses and other abnormalities of the femur and the innominate bone. However, the changes of the skeleton observed in this study were obtained through denervation atrophy of the skeletal muscles related to them. The changes observed may, therefore, be considered direct results of transection of nerves related to them. The observations of this study are, therefore, best presented in four sections as the effects of transection of (1) the nerves supplying the adductor longus muscle, (2) the obturator nerve, (3) the femoral nerve, and (4) the nerves of origin of the lumbar plexus.

Effects of Transection of Nerves Supplying Adductor Longus Muscle.—The animals subjected to section of the nerves supplying the right adductor longus muscle recovered rapidly from the operation, and all remained healthy in appearance throughout the period of observation. For the first few days immediately following the operation all animals exhibited a marked limp, with little weight being placed on the right posterior appendage. The limp and lameness in walking disappeared rapidly, and, after one week of recovery, all differences in the running and walking movements of the right and left posterior appendages had disappeared. The power of adduction for the left and right posterior appendages was determined by grasping the foot, pulling laterally, and noting the strength of jerking movements made in an effort to free the foot. Little or no difference in the strength of jerking movements were noted. For all animals of this group, therefore, compensation for loss of adduction was early established for running and walking and the movements of the denervated right and intact left posterior appendages appeared identical and normal.

Autopsy examination showed the visceral organs of all animals of this group to be grossly normal. Examination also showed that the nerve supply of the adductor longus muscle had been successfully interrupted for all except the two animals noted in the Table. Also, at autopsy examination it was noted that atrophic decrease in size of the right adductor longus muscle was slight for the animal killed one week after nerve section but definite and pronounced for animals killed on the second and following weeks after nerve section was performed. The right adductor longus muscle was normal for the two animals of this experiment for which interruption of the nerve supply to that muscle was not successfully performed. The pectineus muscle was normal for the right thigh of all animals of this group. No signs of atrophy were noted for any of the muscles of the left posterior appendage of the animals of this group.

The purpose of this section of the study was, as noted earlier, to determine the importance of the adductor longus muscle and of the medial portion of the pectineus muscle in stimulating formation of the pectineus-adductor longus exostosis located at their common insertion to the femur. Figure 1, a photograph of the femurs of a rat continued on the lathyrogenic diet for eight weeks, shows the position and nature of a fully developed pectineus-adductor longus exostosis for the femur of the left posterior appendage with intact nerve supply to the adductor longus and pectineus muscles. Also shown in Figure 1, for the same animal the femur of the right posterior appendage with denervated and atrophied adductor longus muscle lacked a pectineus-adductor longus exostosis. However, the right femur of this animal possessed a narrow ridge-like exostosis placed at the insertion of the lateral portion of the pectineus muscle to the medial surface of its shaft. This exostosis, which may be named the pectineus exostosis of the

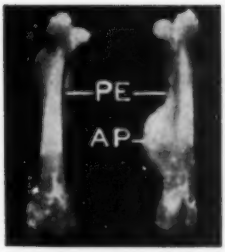

Figure 1

Figure 2

Fig. 1.—Anterior view of right and left femurs of rat fed sweet-pea diet for eight weeks. Nerves to right adductor longus muscle transected. Iliacus-psoas major exostoses removed. *PE* indicates pectineus exostosis; *AP*, pectineus-adductor longus exostosis.

Fig. 2.—Anterior view of right and left femurs of rat fed sweet-pea diet for 11 weeks. Right obturator nerve transected. *IP* indicates iliacus-psoas major exostosis; *PE*, pectineus exostosis; *AP*, pectineus-adductor longus exostosis.

femur, was found to extend from the lesser trochanter to the middle of the shaft. Figure 1 shows that the left femur, in addition to possessing a pectineus-adductor longus exostosis, also possessed a pectineus exostosis represented by a ridge of bone extending from the lesser trochanter to the pectineus-adductor longus exostosis.

Examination of the femurs of all of the animals of this group for which transection of the nerve supply of the right adductor longus muscle had been successfully performed and which had been fed the lathyrogenic diet for two weeks or longer revealed that all left femurs possessed a pectineus-adductor longus exostosis and a pectineus exostosis and that all right femurs possessed only a pectineus exostosis. The two animals with intact nerve supply to both the right and the left adductor longus muscle possessed right and left femurs with a pectineus-adductor longus exostosis and a pectineus exostosis.

Two conclusions may be drawn from the results obtained in this experiment by denervation atrophy of muscles. First, elimination of the pectineus-adductor longus exostosis from the femur of lathyric rats demonstrates that simultaneous stimulation of exostosis-producing tissues by a muscle factor and the Lathyrus factor is required for the formation of exostoses. Second, the results demonstrate that inactivation of muscles by denervation atrophy, thereby preventing formation of an exostosis, provides a satisfactory method of determining the muscle or muscles which produce an exostosis. Two exostoses were identified in this experiment, the pectineus exostosis and the adductor longus exostosis. In advanced stages of development the adductor longus exostosis and the pectineus exostosis become confluent to form the pectineus-adductor longus exostosis. It is clear that the major portion of the pectineus-adductor longus exostosis is produced through the agency of the adductor longus muscle.

Effects of Transection of the Obturator Nerve.—The animals subjected to transection of the obturator nerve recovered rapidly from the operation of nerve section and, like the animals subjected to section of the nerve supply of the adductor longus muscle, appeared healthy throughout the period of the experiment. For the first few days following nerve section the animals of this group exhibited somewhat awkward walking movements. Improvement was rapid, and even though the muscles of the right adductor group were functionally inactive, the movements of the denervated right and the intact left posterior appendages became identical and normal. Compensation for loss of adduction by denervation of the adductor muscles was, therefore, early established for running and walking.

The visceral organs of all animals of this group at autopsy examination were found to be grossly normal. Autopsy examination also showed the right obturator nerve to have been successfully transected for all animals of the experiment. Atrophic decrease in size of the right adductor muscles, excepting the right adductor magnus muscle, which receives double innervation from the obturator and sciatic nerves, varied from barely recognizable decrease for the animal killed one

week after nerve section to very marked decrease in size for animals killed several weeks after nerve section. All muscles of the left posterior appendage were normal for all animals of this experiment.

As preparation for analysis of the results of transection of the obturator nerve on the exostoses of the femur and innominate bone, a brief description of the adductor group of muscles which it supplies will be presented here. The adductor group of muscles of the thigh of the rat include the gracilis anterior, gracilis posterior, adductor longus, adductor brevis, and adductor magnus muscles. All muscles of the group take origin on the pubis parallel to the symphysis pubis and on its lateral surface posterior and inferior to the obturator foramen. The gracilis anterior and gracilis posterior muscles are inserted on the medial surface and crest of the tibia and, therefore, exert no direct influence on the femur. The slender tendon of insertion of the adductor longus muscle and the medial portion of the pectineus muscle, as described in our earlier publication, are attached to the femur near its medial epicondyle.[9] The adductor brevis muscle is inserted on the posteromedial lip or margin of the gluteal trochanter of the femur. The adductor magnus muscle, in addition to being inserted on the medial epicondyle and neighboring region of the femur, is also inserted on the medial surface of the proximal end of the tibia.

Figure 2, a photograph of the right and left femurs of a rat fed the lathyrogenic diet for 11 weeks after transection of the right obturator nerve, except for differences in size of exostoses, may serve as illustration of the femurs of the denervated right and intact left posterior appendages of animals of this group fed the lathyrogenic diet for three weeks or longer periods of time. Though a pectineus-adductor longus exostosis was present and well developed on the left femur, none occurred for the femur of the right appendage of any animal subjected to transection of the right obturator nerve. The gluteal trochanters of both right and left femurs were enlarged, but no thickening had occurred for the posteromedial lip of the gluteal trochanter of the right femur at the insertion of the right denervated and atrophic right adductor brevis muscle. However, thickening and exostosis formation had occurred on the margin and anterior lip at the insertion of gluteus superficialis and gluteus maximus muscles to the gluteal trochanters of both right and left femurs. The distal ends of both the right and left femurs of the animals of this experiment, as shown in Figure 2, were similar and normal. It appears, therefore, that the adductor magnus muscle commonly does not produce an exostosis on the femur.

Exostoses not related to the adductor muscles, as shown in Figure 2, were present and similar for the right and left femurs. The greater and lesser trochanters were enlarged about equally for the right and left femurs. Though not of equal size, the iliacus-psoas major exostoses were large for both femurs. The pectineus exostosis of the femur, described in detail in the first section on results of this study, was present on both right and left femurs. However, the pectineus exostosis of the right denervated appendage of some animals fed the lathyrogenic diet for more than eight weeks was very broad at the distal end and for others at the distal end possessed a superiorly and medially directed spur of bone. The spur-like projection of bone, when of large size, resembled the pectineus-adductor longus exostosis but was distinguished from that exostosis by its more proximal position on the shaft of the femur.

Examination of the innominate bones of lathyric animals killed two weeks after transection of the right obturator nerve revealed but little difference between the right and left innominate bones. Differences between the right and left innominate bones were sufficiently developed to be recognized for animals fed the lathyrogenic diet for

three weeks and prominent for animals fed the diet for four weeks or longer. An exostosis at the origin of the pectineus muscle was present on the pubis of both innominate bones but for all animals was much larger for the innominate related to the denervated right appendage than for the innominate related to the left intact appendage. The left pubis parallel to the symphysis pubis was found to be thickened at the origins of the functionally active adductor longus and adductor magnus muscles. However, the right pubis at the origin of the right denervated and atrophied adductor brevis and magnus muscles was very thin and slender. A very large exostosis was present at the origin of the quadratus femoris on the ischium of the right innominate bone. An exostosis was also present at the origin of the left quadratus femoris muscle; it was of small size for all animals.

The effects of transection of the right obturator nerve and the resulting atrophy of the adductor muscles on the exostoses of the right femur and innominate bone of lathyric rats may be summarized by the following statements. For the femur, denervation and atrophy of the adductor muscles prevented formation of the pectineus-adductor longus exostosis and prevented formation of the adductor brevis portion of the exostosis of the gluteal trochanter. Absence for several weeks of functional adductor muscles caused an increase in size and structural modification of the right pectineus exostosis to occur, thereby indicating that the pectineus muscle had assumed much of the work of adduction of the right posterior appendage. For the right innominate bone, inactivation of the adductor muscles prevented thickening of the pubis at the origin of the adductor longus and adductor magnus muscles. The large size of the exostosis on the pubis at the origin of the pectineus muscle provides further evidence that the pectineus muscle assumes the function of adduction lost by denervation of the adductor muscles. The large size of the exostosis at the origin of the right quadratus femoris muscle, when compared to the exostosis at the origin of the left quadratus femoris muscle, suggests that the right quadratus femoris may have assumed some of the functions normally performed by the denervated right adductor muscles. Observations on compensatory changes related to the pectineus and quadratus femoris muscles assume greater significance when it is recalled that running and walking movements for the animals of this group were normal after recovery from the operation of nerve section.

Effects of Transection of the Femoral Nerve.—Observations on the influence of muscles denervated by transection of only the femoral nerve are limited to those made on the one animal identified in the Table. The changes of the skeleton noted for this animal will be given separate consideration because information on the changes which follow femoral nerve section will be of assistance in interpretation of the results of interruption of the lumbar plexus to be described in the next section.

The animal recovered rapidly from the operation of right femoral nerve transection, but when examined nine weeks later it was found to be lame and walking movements were found to be awkward for the right posterior appendage. In walking, the right leg was rigidly flexed at the knee; the foot was extended, and the weight of the body was placed on the toes of the foot. Movements of the hip joint, ankle joint, and the joints of the foot were normal. Transection of the right femoral nerve, therefore, resulted in permanent disability of the right posterior appendage.

At autopsy examination, all visceral organs, except the right kidney and ureter, grossly presented a normal appearance. The right kidney was enlarged and hydronephrotic, and the proximal end of the right ureter was dilated. The enlargement of the right

kidney and ureter was caused by mechanical injury to the ureter during the operation of nerve section. The right pectineus, quadriceps femoris, and iliacus muscles were markedly atrophied. Though the anterior portion of the psoas major muscle was normal in size, the posterior portion of that muscle was found to have undergone atrophic decrease in size. It was also noted at autopsy, and later verified by dissections of the preserved carcass, that the right femoral nerve had been sectioned proximal to the origin of its branches to the iliacus muscle and to the posterior portion of the psoas major muscle.

Figure 3 is a photograph of the right and left femurs of the animal subjected to section of the right femoral nerve. The femur of the left posterior appendage with intact femoral nerve and functionally active muscles was found to present a typical lathyrism pattern of exostoses. The greater and lesser trochanters were enlarged. The gluteal trochanter possessed an exostosis expanded in both anterior and posterior directions. The iliacus-psoas major exostosis and pectineus-adductor longus exostosis were present and well developed. The distal end of the femur appeared unchanged and normal.

Fig. 3.—Anterior view of right and left femurs and ventral view of innominate bones of rat fed sweet-pea diet for nine weeks. Right femoral nerve transected at origin from lumbar plexus. *PE* indicates pectineus exostosis; *Q*, quadratus femoris exostosis; *IP*, iliacus-psoas major exostosis; *X*, stump of atrophied right iliacus and psoas major muscles; *G*, exostosis of gluteal trochanter; *AE*, adductor longus exostosis; *D*, general enlargement.

Three prominent modifications of the exostosis pattern characteristic of the femur of lathyric rats were present on the femur of the right posterior appendage of this animal. First, as shown in Figure 3, denervation and consequent atrophy of the iliacus muscle and posterior portion of the psoas major muscle by section of the femoral nerve prevented formation and development of the iliacus-psoas major exostosis. Second, denervation and atrophy of the pectineus muscle prevented formation of the pectineus exostosis. The third change was related to the areas of origin from the femur of the denervated and atrophied quadriceps femoris muscle. Deposits of bone at the origins of this muscle cause a thickening and increase in diameter of the femur of lathyric rats with active functioning muscles. For the right femur an increase in diameter was prevented from occurring by denervation and atrophy of the right quadriceps femoris muscle. Compare the right and left femurs of this animal, shown in Figure 3, for differences in diameter of the shaft.

A fourth change of the right femur of this animal we believe requires special notice, namely, marked enlargement and distortion of the distal end. Enlargement of the distal end of the femur of adult lathyric rats, in our experience, is of infrequent occurrence. As shown in Figure 3, the condyles and epicondylar region of the right femur are larger than the comparable region of the left femur. Also, the increase in size and distortion is greater for the medial than for the lateral condyle and epicondyle. Since the changes observed were present only on the right femur, with no comparable change

for the left femur, they must in some manner be related to the only muscle of this region which had been denervated and had atrophied, namely, the quadriceps femoris muscle.

The adductor muscles of the right as well as of the left posterior appendage were normal and functionally active and, as shown in Figure 3, produced exostosis for the right as well as the left femur. An exostosis was present at the insertion of the adductor longus muscle, and, because a pectineus exostosis was not present, the exostosis at the insertion of the adductor longus muscle was a true adductor longus exostosis, produced under the sole influence of that muscle. Also, because the adductor brevis muscle of the right appendage was normal, the right femur possessed a large gluteal trochanter exostosis, a great part of which was produced under the influence of that muscle. Though the right adductor magnus muscle produced no change of the distal end of the left femur, it is possible that the right adductor magnus muscle, in the absence of a functional right quadriceps muscle, may have produced or participated in producing the enlargement and distortion of the distal end of the right femur.

Denervation and atrophy of the right iliacus muscle and the pectineus muscle produced prominent changes of the exostosis pattern of the right innominate bone. First, because the area of origin on the ilium of the iliacus muscle is narrow and bordered by origins of other muscles, the inferior surfaces of the right and left ilium, as shown in Figure 3, are closely similar. Second, whereas a very large exostosis was present on the left pubis at the origin of the normal left pectineus muscle, denervation and atrophy of the right pectineus muscle prevented formation of an exostosis at its origin on the right pubis. Third, the right and also the left adductor longus and adductor magnus muscles, supplied by intact right and left obturator nerves, produced thickening of both the right and left pubic bones. However, thickening was considerably greater for the right than for the left pubis, thereby indicating that the right adductor muscles had assumed some of the functions of the atrophied right pectineus muscle. Fourth, the exostosis at the origin from the ischium of the quadratus femoris muscle was much larger for the right than for the left side. It appears, therefore, that the right quadratus femoris may have assumed some of the functions of the atrophied right pectineus muscle.

Briefly summarized, transection of the femoral nerve and resulting denervation and atrophy of the quadriceps femoris, pectineus, and iliacus muscles and a portion of the psoas major muscle resulted in the formation of an atypical lathyric femur. Iliacus-psoas major and pectineus exostoses were absent, and as a result of the absence of the pectineus exostosis, an exostosis produced at the insertion of the adductor longus muscle was a true adductor longus exostosis. Thickening of the shaft by the quadriceps femoris was absent. The condyle and epicondylar regions were enlarged and misshapen. Enlargement of the greater, lesser, and gluteal trochanters occurred in the usual manner. Also, the exostosis pattern of the innominate bone was changed by elimination of the pectineus exostosis. The pubis at the origin of the right adductor longus and magnus muscles was thickened more than usual for lathyric rats. The greater than usual thickening of the pubis and the greater than usual size of the exostosis at the origin of the right quadratus femoris muscle indicate that the functions of the atrophied pectineus muscle possibly were assumed largely by the adductor longus, adductor magnus, and quadratus femoris muscles.

Effects of Transection of Nerves of Origin of Lumbar Plexus.—All animals subjected to interruption of the nerves of origin of the right lumbar plexus, on recovery from the operation of nerve section, early acquired a peculiar swinging gait which continued, without modification, throughout the experiment. The swinging gait appeared to be due to abnormal position and limited

movement of some of the segments of the right posterior appendage. Movement of the hip joint was limited, but movements at the ankle and various joints of the foot were normal. Very little or no movement occurred at the knee joint, and the leg was fixed in the position of flexion on the thigh. When the weight of the body was transferred to the right appendage, shortened by fixed flexion at the knee, the body swung far to the right. In walking, the body of these animals swung from side to side to produce a wobbling type of gait. The animals of this group did not run but hopped when moving rapidly. Section of the nerves of origin of the lumbar plexus was, therefore, followed by marked and permanent changes of locomotion.

No abnormalities of the visceral organs, excepting the right kidney and ureter for several animals, were noted at autopsy examination. For some of the animals continued on the lathyrogenic diet for three weeks or longer, the right kidney was greatly enlarged, while the left kidney was of normal size and structure. The enlarged right kidneys were found to be hydronephrotic. Dilatation, terminating at a constriction of the wall, of the proximal portion of the right ureter also was present. Since the genitofemoral nerve and the ureter lie side by side throughout a great part of their course, the right ureter undoubtedly was injured by surgical manipulations related to locating lumbar Nerve 2.

It was shown by autopsy examination, and later confirmed by dissection of the preserved specimens, that the anterior divisions of lumbar Nerves 2, 3, and 4 had been transected. The degree of atrophy of the muscles of the undissected right posterior appendage of an animal killed eight weeks after nerve section is shown in Figure 4. The muscles of the right posterior appendage deprived of their nerve supply were the iliacus, psoas major, quadriceps femoris, pectineus, and the members of the adductor group, namely, the adductor longus, adductor brevis, adductor magnus, gracilis anterior, and gracilis posterior. The adductor magnus, because of its double innervation through the sciatic and obturator nerve, when compared to other muscles showed only a moderate decrease in size.

Fig. 4.—Ventral view of posterior appendages and pelvis of rat fed sweet-pea diet for eight weeks. Nerves of origin of right lumbar plexus transected. *PM* indicates normal psoas major muscle; *QF*, atrophied quadriceps femoris muscle; *P*, right atrophied and left normal pectineus muscles; *A*, right atrophied and left normal adductor longus muscles.

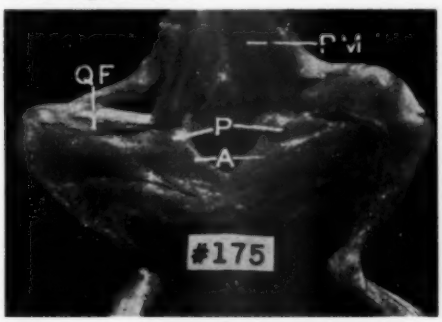

Fig. 5.—Anterior view of right and left femurs and ventral view of innominate bones of rat fed sweet-pea diet for 10 weeks. Nerves of origin of right lumbar plexus transected. *PE* indicates pectineus exostosis; *N*, normal right pubis; *Q*, quadratus femoris exostoses; *IP*, iliacus-psoas major exostosis; *AP*, pectineus-adductor longus exostosis; *D*, general enlargement.

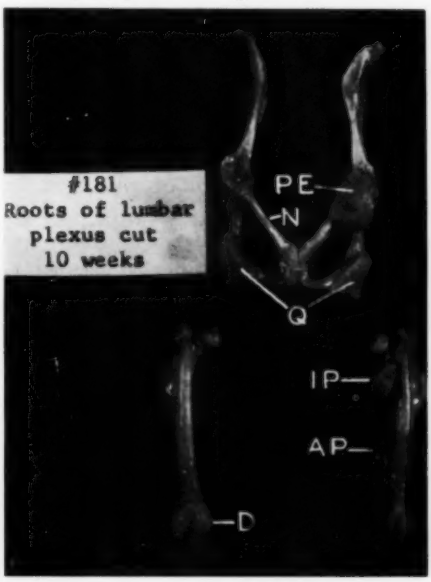

Figure 5, a photograph of the femurs of the denervated right and intact left posterior appendages of a rat fed the lathyrogenic diet for 10 weeks, shows that origin and development of the iliacus-psoas major, pectineus, and pectineus-adductor longus exostoses was prevented by denervation of the muscles related to them. The adductor brevis portion of the gluteal trochanter exostosis was absent, thereby decreasing the size of that exostosis. Also, thickening of the shaft of the femur at the origins of the vastus muscles was prevented by denervation of the quadriceps muscle.

The right femur of animals subjected to interruption of the lumbar plexus and fed the lathyrogenic diet for several weeks presents a fairly normal nonlathyritic appearance. However, because of the influence of muscles innervated through the sciatic plexus, the greater trochanter, the lesser trochanter, and the gluteal division of the third trochanter were larger than normal. Also, the distal end of the right femur exhibited an enlargement of the medial condyle and epicondyle. The enlargement of the distal end of the femur appears to be the same phenomenon observed after section of the femoral nerve. The loss of tension supplied by the quadriceps muscle and the tension applied to the medial side by the tenuissimus and partially atrophied adductor magnus muscles must in some manner help to produce the enlargement of the distal end of the femur.

The exostosis pattern of the right innominate bone, like the pattern of the right femur, was markedly altered by transection of the roots of the right lumbar plexus. As shown in Figure 5, the right ilium was narrow and smooth at the origin of the atrophied iliacus muscle. An exostosis failed to develop on the pubis at the origin of the atrophied right pectineus muscle. Also, the pubis failed to thicken at the origin of the atrophied right adductor longus and adductor magnus muscles. The only exostosis, other than those related to the gluteal muscles, present on the right innominate bone was the large exostosis at the origin of the right quadratus femoris muscle. In contrast, the innominate bone related to the left posterior appendage with intact nerves and functional muscles was roughened at the origin of the iliacus muscle, possessed a large exostosis at the origin of the pectineus muscle, and presented a fairly marked thickening of the pubis at the origin of the adductor longus and magnus muscles. A quadratus femoris exostosis of moderate size was present on the left innominate bone.

By causing denervation atrophy of the large number of muscles supplied by the obturator and femoral nerves through the lumbar plexus, it was possible in this experiment to prevent formation of the major exostoses and abnormalities of both the femur and the innominate bone. By preventing formation of the major exostoses, the femur and innominate bone of the right side were made to present a practically normal appearance, while the left femur and innominate bone of the same animal presented the full complement of exostoses and abnormalities commonly seen in lathyric rats.

Comments

Though a number of investigators of lathyrism of the rat have noted that exostoses appear to form at points of attachment of muscles to the skeleton, only a small number of investigators have attempted to identify exostoses with specific skeletal muscles.[6,9,10] The present study, by a series of denervation experiments, has demonstrated that all exostoses, or other abnormality of the skeleton investigated, are dependent on one or more muscles for their formation. Further, by denervation of groups of muscles, this study has demonstrated that muscle-group patterns of exostoses occur for the femur and the innominate bone. These observations indicate that further studies very likely will show other exostoses and other abnormalities of the skeleton of lathyric rats to be related to and produced by one or more skeletal

muscles. It is suggested, therefore, that, whenever appropriate, abnormalities of the skeleton of lathyric rats be identified by the name of the muscle or muscles which produce them.

In an earlier publication, we suggested that the exostosis-producing agent or factor of sweet-pea seeds, when acting alone on periosteal tissues, does not cause active production of exostoses and can produce exostoses only in the presence of a second stimulating agent.[9] By demonstrating that transection of muscles prevented formation of exostoses related to them, muscles were shown to serve in some manner as the second factor required for exostosis production by lathyric rats.[10] The present study, by preventing formation of the major exostoses of the femur and innominate bone through inactivation of the muscles related to the exostoses, confirms the observation that the Lathyrus factor and the muscle factor must simultaneously stimulate periosteal tissues if production of exostoses is to occur. By employing denervation atrophy of muscles to prevent formation of exostoses, this study has also demonstrated that only active muscles, undoubtedly through tension applied to periosteal tissues, can serve as the second factor for production of exostoses. It is interesting to note that, like the Lathyrus factor, aminoacetonitrile can produce exostoses only in the presence of functionally active muscles.[13] It must be concluded that experiments on lathyritic abnormalities of the skeleton must be interpreted on the basis of the condition of the skeletal muscles as well as on the presence or absence of the Lathyrus factor.

Reference in this paper has frequently been made to the phenomenon of compensatory formation of new exostoses or the increase in size of commonly occurring exostoses. These changes appear to occur when one muscle assumes the function lost by inactivating some neighboring muscle or muscles. Several prominent instances of this phenomenon were observed in this study. First, the exostosis on the pubis at the origin of the pectineus muscle was found to be much larger than usual when the adductor muscles of the same side were atrophied. Second, the opposite condition of greater development of the thickening of the pubis at the origin of the adductor longus and magnus muscles occurred when the pectineus muscle was inactive. The third, also associated with adduction of the thigh, was shown by the greater size of the quadratus femoris exostosis on the ischium of the side of atrophied adductor muscles than for the opposite side where the adductor muscles were intact. Fourth, a spur-like prominence developed at the distal end of the pectineus exostosis when, after inactivation of the adductor muscle, the pectineus muscle alone served adduction. Fifth, the enlargement of the medial side of the distal end of the femur under the possible influence of the adductor magnus and tenuissimus muscles, after denervation of the quadriceps femoris muscle, may serve as an example of compensatory development of a new skeletal abnormality. The examples cited provide satisfactory evidence of the influence of compensation on production of abnormalities of the skeleton in lathyrism.

Other investigators have pointed out that an abnormal type of development of epiphyseal cartilages occurs regularly for young lathyric rats.[6] We believe the enlargement of the distal end of the femur of adult lathyric rats observed by us to follow section of the femoral nerve or nerves of origin of the lumbar plexus, and shown in Figures 3 and 5, is an example of the same phenomenon. It is important to note that the enlargement of the distal end of the femur of adult lathyric rats occurs regularly only in the presence of an atrophied quadriceps femoris muscle. The enlargement of the distal end of the femur was marked when the adductor magnus and tenuissimus muscles, inserted on that region of the femur, were intact and functional. However, when the adductor magnus muscle was partly atrophied and only the tenuissimus normal and active, the enlargement of the

distal end of the femur was moderate or slight. It appears, therefore, that muscle tension is an important factor in the production of that abnormality. We suggest that abnormal development of epiphyseal cartilages, like the formation of exostoses, is due to the combined stimulation of those structures by the Lathyrus factor and the muscle-tension factor.

The observations made in this study have been employed to solve the practical problem of the innervation of two muscles of the pelvis and thigh of the rat. One author states that the quadratus femoris muscle is innervated through the obturator nerve,[11] but that cannot be true because transection of that nerve neither causes atrophy of that muscle nor prevents exostosis formation by that muscle. The quadratus femoris muscle of the rat, like that of man, is supplied through the sciatic plexus. Also, the pectineus muscle is stated to be supplied by both the obturator and femoral nerves. Transection of the obturator nerve did not cause atrophy of that muscle or interfere in any way with the production of exostoses by that muscle. Transection of the femoral nerve, however, caused the pectineus muscle to atrophy and prevented formation of its exostoses. For our animals, therefore, the pectineus muscle was innervated solely by the femoral nerve. Because of its usefulness in solving the problem of innervation of the two muscles, a technique of employing lathyrism and transection of nerves may possibly be of value in solving problems related to the skeleton, muscles, and nerves.

Summary

Thirty-five adult female rats with an average body weight of 249 gm. and ranging to one year of age were employed in this study of the role of skeletal muscles in the production of exostoses and other abnormalities of the skeleton by lathyric rats. The method of inactivation of muscles by denervation was employed, and, to permit each animal to serve as its own control, transection of the nerve supply to muscles commonly associated with skeletal abnormalities was performed for only the right posterior appendage of each animal. Nerves transected were either the nerve supply to the adductor longus muscle, the obturator nerve, the femoral nerve, or the nerves of origin of the lumbar plexus. After nerve section, all animals were given a diet composed of equal parts of meal of Purina Laboratory Chow and meal of seeds of the sweet pea Lathyrus odoratus. The animals were killed for study after varying periods on the diet.

Denervation, and consequent atrophy, of muscles of the right posterior appendage in all cases prevented formation on the femur of exostoses or other skeletal abnormalities related to those muscles. Section of the nerve supply of the adductor longus muscle prevented formation of the pectineus-adductor longus exostosis located at the combined insertion of the medial division of the pectineus muscle and the adductor longus muscle. Thereby a pectineus exostosis produced by the lateral division of the pectineus muscle was isolated and identified. Section of the obturator nerve prevented formation of the pectineus-adductor longus exostosis and the adductor brevis portion of the exostosis of the gluteal trochanter. Section of the femoral nerve proximal to the origin of branches to the iliacus muscle and posterior region of the psoas major muscle prevented formation of the iliacus-psoas major exostosis and pectineus exostosis and thickening of the cortex at the origin of the quadriceps femoris muscle. As a result, a true adductor longus exostosis was isolated and identified. An unusual enlargement of the distal end of the femur followed transection of the femoral nerve. Transection of the nerves of origin of the lumbar plexus produced practically a normal nonlathyritic type of femur by preventing formation of exostoses related to both the obturator nerve and the femoral nerve. The only abnormalities remaining after interruption of the lumbar plexus were enlargements of the greater trochanter, the lesser trochanter, and the

gluteal portion of the exostosis of the gluteal trochanter and enlarged and misshapen medial condyle and epicondyle.

Denervation and atrophy of muscles also prevented formation of exostoses and other abnormalities of the right innominate bone of lathyric rats. Transection of nerves to the adductor longus muscle had no marked effect on the right innominate bone. Section of the obturator nerve prevented thickening of the pubis at the origin of the adductor longus and magnus muscles but increased the size of the exostoses at the origins of the right pectineus and quadratus femoris muscles. Section of the femoral nerve prevented thickening of the ilium at the origin of the iliacus muscle and formation of the pectineus exostosis on the pubis. However, compensatory increased thickening of the pubis occurred at the origin of the adductor longus and magnus muscles. Increase in size of the exostosis at the origin on the ischium of the quadratus femoris muscle was also present. Section of the nerves of origin of the lumbar plexus, by causing atrophy of the iliacus, pectineus, and adductor muscles, prevented formation of all abnormalities of the ventral and lateral surfaces of the innominate bone except the quadratus femoris exostosis and thickenings related to the origin of the gluteal muscles.

This study demonstrates that all abnormalities of the femur and innominate bone can be identified with specific skeletal muscles. This study also demonstrates that all abnormalities of those bones are not formed by lathyric rats when the muscles related to them through denervation atrophy are functionally inactive. Those observations conclusively prove that formation of abnormalities of the skeleton by the active agent of sweet peas can occur only in the presence of a second factor of muscle tension supplied by functionally intact skeletal muscles.

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Effects of Gossypol in Young Chickens with the Production of a Ceroid-like Pigment

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The effects of gossypol* have been studied in the cat,[1] dog,[2,3] mouse,[1,4] rat,[1,4-7] rabbit,[1,4,8] hog,[9-11] guinea pig,[1,4] chicken,[10,12,13] and fish.[14] In a study of chronic intoxication in cats and rabbits, Schwartze[1] observed a loss of appetite, paralysis with nerve degeneration, shortness of breath, edema of the lungs, and an effusion into the serous cavities. West[2] observed that death occurred in dogs within one to two days when 27% cottonseed meal was put into the diet of weaning pups. In these animals the lungs and liver were ecchymotic, the viscera were edematous, ulcers were present in the stomach and proximal portions of the intestines, and focal areas of necrosis were present in the liver. Eagle et al.[4] also observed hemorrhages in the intestines of rats, mice, guinea pigs, and rabbits fed gossypol. Ambrose and Robbins,[5] however, failed to find any evidence of tissue damage in rats fed gossypol. It is interesting to know that Ambrose and Robbins[5] observed a finely granular, dark pigment in the Kupffer cells of the liver that gave a negative Prussian blue reaction. Hale[9] and Smith[11] observed edema and congestion of the viscera in hogs fed gossypol. The heart was hypertrophied in some of the animals, and the liver had extensive degeneration about the central veins. Effusion was frequent in the serous cavities.

Menaul,[14] in 1923, studied the effect of gossypol on the liberation of oxygen from sheep erythrocytes. He found that gossypol inhibited the liberation of oxygen from hemoglobin and hemolyzed sheep erythrocytes in vitro. Menaul[14] expressed the opinion that "gossypol causes death in animals by reducing the oxygen-carrying capacity of the blood."

We have studied the effect of gossypol on young chicks, with emphasis on the red blood cells and on the histologic changes occurring in the viscera. Our observations are reported at this time.

Methods and Materials

Hematologic studies were made at the end of four weeks on 140 Hyline White Leghorn chicks. Erythrocyte counts were made with use of standard techniques and dilution of the blood with Gower's solution.[15] The hemoglobin was determined according to the method of Sanford et al.[16] The chicks when one day of age were divided into

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* Gossypol is found in the gossypol glands in cottonseed and is present in commercial meal obtained by crushing cottonseed. The chemical composition of gossypol is included in the publication by Bailey.[10]

seven groups of 20 each. They were given a basic diet containing 4.0% total fats, 6.0% salts IV,* and 50% cottonseed meal, which supplied 21% crude protein and 40% glucose monohydrate. Adequate amounts of vitamins were added to the diet. Gossypol in the form of pigmented glands obtained from cottonseed was added to the basic diet in the following concentrations: 0.01%, 0.03%, 0.04%, 0.05%, 0.06%, 0.07%, and 0.15%. These chicks were kept in electrically heated batteries with raised screen floors. Feed and water were given ad libitum. The birds were weighed at weekly intervals. In subsequent studies, the chicks were fed the purified synthetic type chick diet of Reid and co-workers.[17]

Red blood cell counts were made on a second group of 90 White Rock chicks fed gossypol in the form of pigment glands. These chicks when one day of age were divided into six equal-sized groups. The amount of gossypol given the chicks in each group is shown in Table 2. After nine weeks, blood for erythrocyte counts was removed from the wing vein of either three or four of the chicks in each group.

Pathologic studies were made on 10 New Hampshire Red chicks fed a diet containing 0.4% gossypol in the form of pigment glands. Only chicks killed and autopsied immediately were used. Bone marrow smears were air-dried and fixed immediately in methyl alcohol and stained with Wright-Giemsa dye. Five hundred cells were counted in each smear, and the average was recorded. Two chicks were killed when 8 days of age; five, when 15, and three, when 22 days of age. Eleven chicks of the same age and breed were killed for controls. The tissues were fixed immediately in a 4.0% solution of formalin. Paraffin sections were prepared and stained routinely with hematoxylin and eosin. Select sections were stained by the following techniques: Periodic acid Schiff, osmic acid, oil red O, Mallory's method for hemofuscin, Hucher-Conn ammonium oxalate methylrosaniline chloride (crystal violet), Prussian blue reaction for iron, Kinyoun acid-fast, and Galantha stain for mucin.

Results

The amount of hemoglobin and the number of erythrocytes in the chicken progressively decreased with the increase in the amount of gossypol in the diet, as shown in Tables 1 and 2. The weight of these birds also decreased with the increase in concentration of gossypol. Table 3 gives the percentage of erythroid and myeloid cells in the femur bone marrow of the gossypol-treated and the normal chick. Although the number of observations is too few for a statistical study, these data show that the combination of a progressively falling peripheral red cell count and an erythroblastic hyperplasia of the bone marrow is consistent with a hemolytic process. Normal chicks, as they increase in age, have a progressive decrease in the number of erythroid cells in the bone marrow. Chickens of the same age when given gossypol have a slower decrease in the number of erythroid cells than do the

TABLE 1.—*The Effect of Free Gossypol on Hemoglobin and Erythrocytes in Young Chicks* *

Free Gossypol in Diet, %	Hemoglobin Content		Erythrocyte Count		Weight, Gm. 4-Wk.
	Gm./100 Ml.	% Normal	Millions/1 CuMm.	% Normal	
0.01	9.61 †	100.0	2.38	100.0	260.0
0.03	8.51	88.6	2.41	101.3	225.2
0.04	8.28	86.2	1.92	80.7	199.4
0.05	8.09	84.2	1.88	79.0	168.8
0.06	7.91	82.3	1.89	79.4	128.1
0.07	7.25	75.4	1.74	73.1	117.4
0.15	5.82	60.6	1.52	64.0	61.7

* Hemoglobin content per cell = $\frac{\text{gm. hemoglobin in 1 ml. of blood}}{\text{no. erythrocytes in 1 ml. of blood}}$

† Normal or control chicks.

TABLE 2.—*Effect of Feeding Gossypol Glands on Red Blood Cell Counts in Growing Chicks*

Group No.	Gossypol Level, %		RBC (9 Wk.) *
	1-3 Wk.	3-9 Wk.	
1	0.02	0.25	1.92
2	0.04	0.30	1.69
3	0.06	0.35	1.85
4	0.08	0.40	1.79
5	0.10	0.10	1.94
6	----	----	2.73

* Average of 3 or 4 per group (millions/cu. mm.).

TABLE 3.—*Effect of Gossypol on Percentage of Erythroid and Myeloid Cells in Bone Marrow of Young Chicks*

Age, Days	% Cells in Bone Marrow			
	Normal		Gossypol-Treated	
	Erythroid	Myeloid	Erythroid	Myeloid
8	47.8	51.6	54.9	44.9
15	29.2	70.5	39.5	60.0
15	33.5	66.6	38.6	61.4
22	25.8	74.0	49.0	50.5
22	23.5	76.4	46.0	53.3

* 600g. $CaCO_3$, 645g. K_2HPO_4, 150g. $CaHPO_4 \cdot 2H_2O$, 204g. $MgSO_4 \cdot 7H_2O$, 335g. NaCl, 55g. $Fe(C_6H_5O_7)_2 \cdot 6H_2O$, 1.6g. KI, 10g. $MnSO_4 \cdot 4H_2O$, 0.5g. $ZnCl_2$, 0.6g. $CuSO_4 \cdot 5H_2O$.

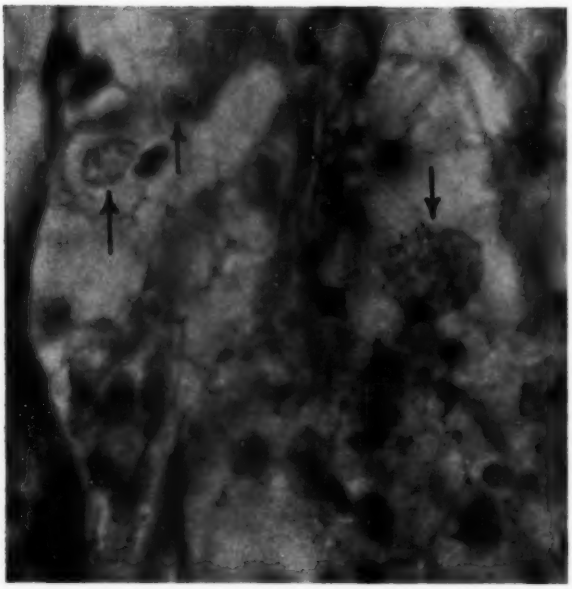

Fig. 1.—Masses of yellowish-brown pigment are present in the duodenal villi of the gossypol-fed chicks. This pigment appears to be free within the lumen of small channels. Hematoxylin and eosin; × 1280.

controls during the first two weeks of the experiment (Table 3). The number of red cell precursors on the 14th day is increased in the gossypol-treated chicks over that in the normal. This variation in the percentage of cells in the bone marrow suggests that the erythroblastic series has been stimulated by some hemolytic mechanism.

No macroscopic changes were observed in the viscera of the 10 gossypol-treated chickens; however, microscopic lesions were present in the intestines, spleen, and liver. Some of the epithelial cells covering the villi in the duodenum either were absent or showed acute degenerative changes in a few of the gossypol-fed chickens. How many of these changes in the intestines resulted from the effect of gossypol and how many resulted from autolysis could not be satisfactorily determined. All tissues, however, were immediately removed and fixed in a 4.0% solution of formalin to prevent as much autolysis as possible. In the hematoxylin-and-eosin-stained sections of the intestine small masses of light yellowish-brown pigment were present in the stroma of the villi in all the chickens receiving the gossypol; none of this pigment was observed in the villi of the controls. The pigment appeared to be free within the lumens of small channels in the duodenal villi that resembled lymphatic or capillary vessels. (Fig. 1).

There was an apparent increase in the number of mucin-secreting epithelial cells, as shown by both the mucin and periodic acid-Schiff staining techniques, in the duodenal villi of the birds receiving the gossypol over that observed in the controls. However, the amount of this increase was not marked.

A yellowish-brown pigment, like that in the duodenal villi, was present in the sinusoids of the spleen (Fig. 2). The size of the individual masses and the quantity of pigment in the spleen increased with the increase in the length of time that the chickens received gossypol. A majority of this pigment appeared to be extracellular within the splenic sinuses.

Many vacuoles were present in the hematoxylin-and-eosin-stained sections of liver (Fig. 3). These varied in size and they occurred throughout the hepatic lobule. These foamy-like spaces were surrounded

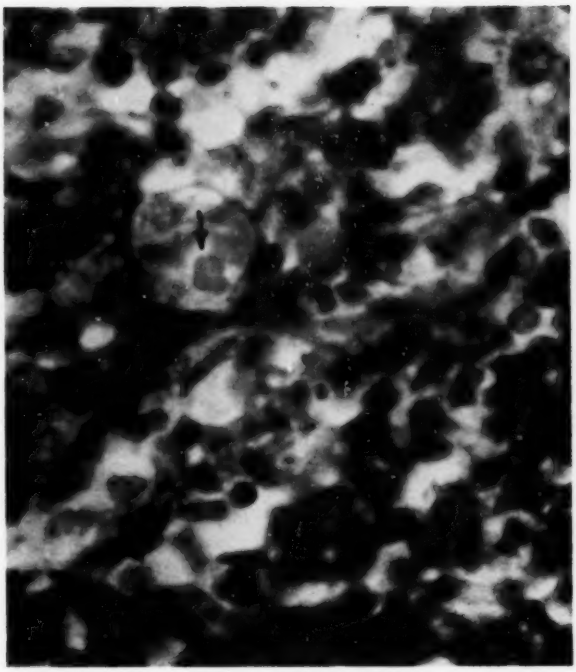

Fig. 2.—A yellowish-brown pigment is present in the sinusoids of the spleen in the gossypol-fed chicks. This pigment gives a positive periodic acid-Schiff reaction and stains positive with the oil red O dye for fat. This pigment gives a negative reaction for iron. Hematoxylin and eosin; × 1280.

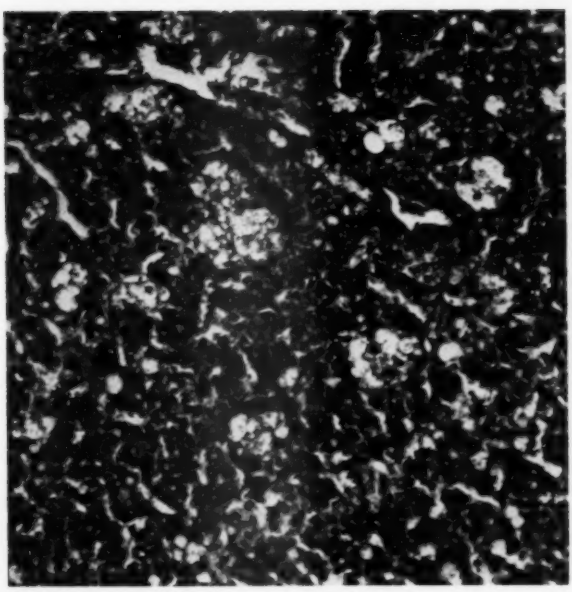

Fig. 3.—Foamy spaces are present in the liver of gossypol-fed chicks. Such areas are the location of lipid pigment in frozen sections. Hematoxylin and eosin; × 220.

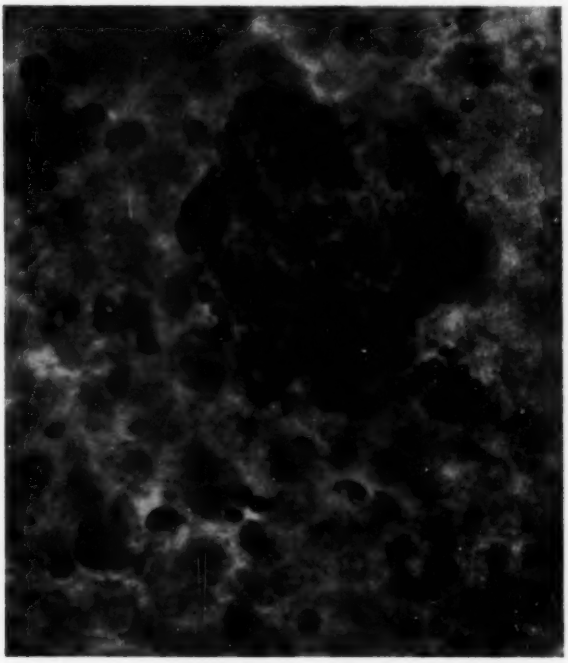

Fig. 4.—Local accumulations of globules of lipid material in the liver of gossypol-fed chicks. Oil red O; ×912.

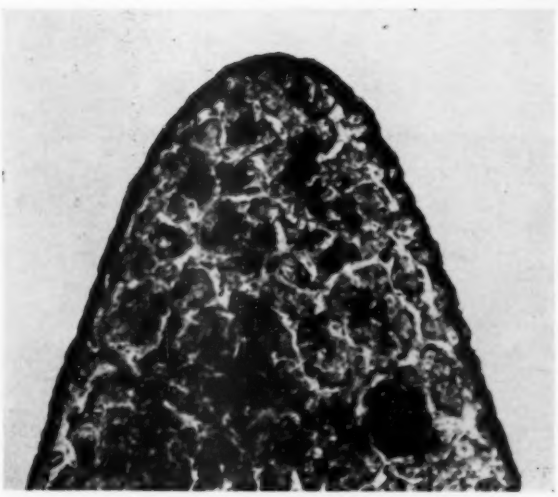

Fig. 5.—Masses of lipid material of different size occur throughout the hepatic lobule of the gossypol-fed chicks. There is a wide variation in the size of the individual globules of fat. Osmic acid; ×220.

by well preserved hepatic cells. These spaces usually appeared to be within hepatic sinusoids, although some were thought to be within the hepatic cords. A few small masses of yellowish-brown pigment were present in the foamy spaces in sections of liver stained with hematoxylin and eosin. Similar masses of pigment also were present in the Kupffer cells.

The clear foamy spaces in the liver, as observed in the hematoxylin-and-eosin-stained sections, represent the location of lipid, as indicated by the oil red O stain (Fig. 4). The lipid material when stained

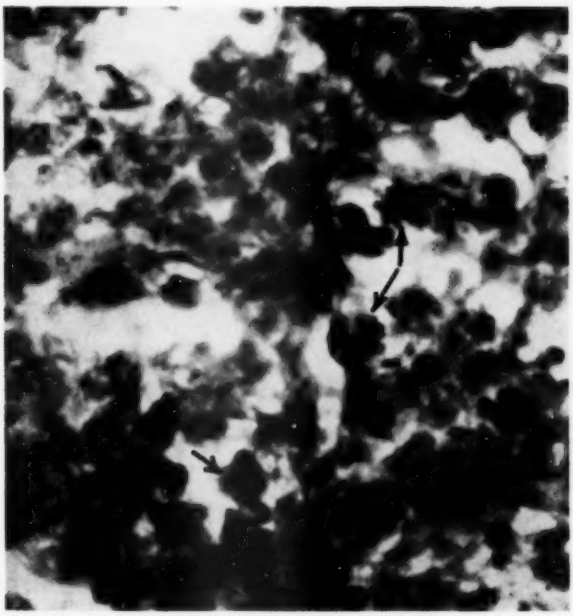

Fig. 6.—The pigment in the spleen of gossypol-fed chicks gives a positive periodic acid-Schiff reaction. Periodic acid-Schiff; × 1280.

in frozen sections by the oil red O technique appeared as dark red globules; it appeared as black granular material when stained with osmic acid (Fig. 5). Only a small amount of pigment was present in the foamy areas of paraffin prepared sections of the liver when compared with the amount of lipid positive-staining material in frozen sections of the liver stained with oil red O.

This pigment in the spleen and liver stained either red or reddish-purple with the periodic acid-Schiff reagent (Fig. 6). Apparently the same pigment that stained positive with the Schiff reagent stained either reddish-brown or brown with the acid-fast stain, bright red with the Mallory's stain for hemafuscin, red with the Hucher-Conn ammonium oxalate methylrosaniline chloride, and negative for iron with both potassium ferric and ferrocyanide stains. All of the lipid-staining pigment was removed in the preparation of the paraffin sections and in some of the staining techniques.

Comment

A hemolytic anemia occurs in chicks fed gossypol. This anemia develops when the diet contains as little as 0.03% free gossypol. At the end of four weeks chicks receiving 0.01% free gossypol, which is considered by us to be innocuous, weigh on the average 260 gm., while birds receiving 0.15% free gossypol average only 61.7 gm.

Ferric oxide, according to Gallup,[18] when added to the diet of rats does not counteract the effects of gossypol. However, when ferrous ammonium sulfate and ferric citrate are added to the diet in amounts to furnish 3 gm. of iron to 1 gm. of gossypol, rats do show a normal rate of growth. Gallup [18] suggested that the iron combined with the gossypol to form either an insoluble compound or a compound that was absorbed slowly, if at all, from the digestive tract. In view of the hemolytic anemia that chickens develop while receiving gossypol, iron would appear to be an aid in hematopoiesis.

Gossypol when fed to young chicks in a concentration of 0.4% produced death of all birds by the 21st experimental day. Chickens fed this same concentration of gossypol and killed on the eighth day of the experiment had a yellowish-brown pig-

ment, both free and in the Kupffer cells, in the hepatic sinusoids. Similar pigment was present in the spleen and in the duodenal villi.

This yellowish-brown pigment in the viscera of the chick apparently is a ceroid. This type of pigment was first described in 1941, by Edwards and White,[19] in the liver of rats with tumors induced by *p*-dimethylamino-azobenzene (butter yellow). A similar pigment has been found in dietary cirrhosis in rats[20] and in mice treated with carbon tetrachloride.[21] Lee,[22] in 1950, suggested that "the ceroid pigment of the mouse is associated with and perhaps formed as a result of processes involved in necrosis of liver cells. Ceroid pigment of the rat may have a similar origin." In 1950, Hartroft[23] suggested that blood cells may play a role in the formation of ceroid pigment. A pigment that possessed the same histochemical properties as the ceroid in cirrhotic livers of rats fed choline-deficient diets was obtained by Casselman, in 1951,[24] from rat erythrocytes treated with cod liver oil.

The distinctive properties of ceroid pigment by which it is most often identified in tissue sections, according to Casselman,[24] are (1) a golden yellow color when unstained, (2) insolubility in organic solvents, (3) sudanophilia (affinity for fat stains, such as the sudan dyes) demonstrable even in paraffin sections, (4) acid-fastness, (5) basophilia, and (6) fluorescence. The pigment in gossypol-fed chicks (1) is yellowish-brown when stained with hematoxylin and eosin, (2) is partly soluble in alcohol, (3) stains positive with oil red O for fat, (4) stains a dark red with the periodic acid-Schiff reagent, (5) is acid-fast-positive, (6) gives a negative Prussian blue reaction for iron, and (7) stains bright red with Mallory's stain for hemofuscin.

There is no significant amount of necrosis either in the liver or in other viscera in these gossypol-fed chickens. They do develop a hemolytic type of anemia soon after receiving gossypol. In view of these findings, it is suggested that this ceroid-like pigment results from the action of gossypol on the erythrocyte. Casselman[24] pointed out that substances possessing the histochemical properties of ceroid-like substances had been prepared from unsaturated fats, fatty acids, and their esters by autoxidation.

From a review of the pathologic findings as described in different animals receiving gossypol, it would appear that anoxia may be a significant mechanism in death. The morphologic changes as found in the viscera of the gossypol-fed chicks do not appear adequate to account for death. It would seem most likely that gossypol interferes with the oxygen-carrying ability of the hemoglobin. Studies are now being made to establish the mechanism of death in gossypol intoxication.

Summary

Chickens fed gossypol develop a hemolytic type of anemia and have a ceroid-like pigment in the duodenal villi and in the sinusoids of the liver and spleen. No significant pathologic changes were observed in the viscera of these birds. These studies would suggest that gossypol affects the erythrocytes to produce this pigment.

Department of Pathology, The University of Texas—Medical Branch.

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News and Comment

ANNOUNCEMENTS

Grants for Training in Pathology.—The department of pathology, New York University-Bellevue Medical Center, has been awarded about $450,000 for a five-year period to train physicians for research and teaching careers in pathology. The training program, which began July 1, was made possible through a grant from the National Institutes of Health and will be directed by Dr. Lewis Thomas, chairman, department of pathology of the New York University College of Medicine.

There are eight research fellowships available at the level of assistant resident. The stipend ranges from $3600 to $4500 yearly. In addition, two senior fellowships are available for physicians with more advanced training in the basic sciences on anatomical pathology, with a stipend of $7500 annually. Information may be obtained from the Dean, New York University College of Medicine, 550 First Ave., New York 16.

Congress of International Society of Hematology.—The Seventh Congress of the International Society of Hematology will be held in Rome at the Palazzo dei Congressi, EUR, on Sept. 7 to 13, 1958.

The official languages of the Congress will be English, French, German, Italian, and Spanish.

Preliminary program: (1) Immunohematology, (2) Hemorrhagic Disorders, (3) Leukemia, (4) Anemia, (5) Nucleonics, (6) The Spleen and the Reticulo-Endothelial System.

For further information write to Segreteria del VII Congresso Internazionale di Ematologia, Istituto di Patologia Medica, Policlinico Umberto I, Roma (Italia).

The Congress of the International Society of Blood Transfusions will be held at the same Palazzo dei Congressi on Sept. 3 to 6.

Symposium on Fundamental Cancer Research.—The Twelfth Annual Symposium on Fundamental Cancer Research will be held March 6, 7, and 8, 1958, at The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas.

The topic for the Symposium will be "Radiation Biology and Cancer." The first day will be devoted to papers from the staff at M. D. Anderson Hospital which relate to the general symposium subject. The final two days of the program will consist of papers presented by recognized authorities in radiation research.

Chairmen for the radiation biology sessions include Titus C. Evans, Radiation Research Laboratory, University of Iowa College of Medicine; Jacob Furth, Pathology Department, Children's Cancer Research Foundation, Inc., Boston, and Henry S. Kaplan, Radiology Department, Stanford University Medical School, San Francisco. General chairman of the Symposium is Warren K. Sinclair, chief physicist at The University of Texas M. D. Anderson Hospital and Tumor Institute.

PERSONAL

Dr. William Boyd Gives G. Lyman Duff Lecture.—At the 11th annual meeting of the American Society for the Study of Arteriosclerosis, held in Chicago, Nov. 2-4, Dr. William Boyd, of Toronto, gave the first annual G. Lyman Duff Lecture.

Dr. Stanley Hartroft Participates in Symposium on Diabetes.—Dr. Stanley Hartroft, of Washington University, was a member of the panel on Symposium Day on Diabetes Mellitus held in New York City in the Hunter College Playhouse Auditorium on Oct. 12. The Symposium was sponsored by the Clinical Society of the New York Diabetes Association.

Appointment for Dr. F. K. Mostofi.—Dr. F. K. Mostofi has been appointed scientific director of the American Registry of Pathology of the Armed Forces Institute of Pathology.

Appointment for Col. John H. Rust.—Col. John H. Rust has been appointed chief of the Veterinary Pathology Section and the Laboratory Animal Branch of the Armed Forces Institute of Pathology.

Appointment for Dr. Osborne A. Brines.—Dr. Osborne A. Brines, Wayne State University Medical School, was made president of the International Society of Clinical Pathology at its session in Brussels, in 1957.

Award to Dr. Harold L. Stewart.—Dr. Harold L. Stewart, of the National Institutes of Health, recently received the Ward Burdick Award of the American Society of Clinical Pathology at the annual meeting in New Orleans.

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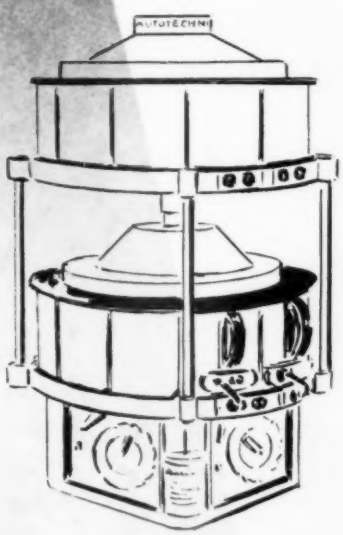

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